(54) Title: COMPOSITIONS AND METHODS FOR DETERMINING ANTI-VIRAL DRUG SUSCEPTIBILITY AND RESISTANCE AND ANTI-VIRAL DRUG SCREENING

(57) Abstract

This invention provides a method for determining susceptibility for an anti-viral drug comprising: (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell; (b) culturing the host cell from (a); (c) measuring expression of the indicator gene in a target host cell; and (d) comparing the expression of the indicator gene from (c) with the expression of the indicator gene measured when steps (a)-(c) are carried out in the absence of the anti-viral drug, wherein a test concentration of the anti-viral drug is present at steps (a)-(c); at steps (b)-(c); or at step (c). This invention also provides a method determining anti-viral drug resistance in a patient. This invention also provides a method for evaluating the biological effectiveness of a candidate anti-viral drug compound. Compositions including resistance test vectors comprising a patient-derived segment and an indicator gene and host cells transformed with the resistance test vectors are provided.

COMPOSITIONS AND METHODS FOR DETERMINING ANTI-VIRAL DRUG SUSCEPTIBILITY AND RESISTANCE AND ANTI-VIRAL DRUG SCREENING

Technical Field

This invention relates to anti-viral drug susceptibility and resistance tests to be used in identifying effective drug regimens for the treatment of viral infections. The invention further relates to novel vectors, host cells and compositions for carrying out these novel anti-viral drug susceptibility and resistance tests. This invention also relates to the screening of candidate drugs for their capacity to inhibit selected viral sequences and/or viral proteins. More particularly, the invention relates to the use of recombinant DNA technology to first construct a resistance test vector comprising a patient-derived segment and an indicator gene, then introducing the resistance test vector into a host cell, and determining the expression or inhibition of the indicator gene product in a target host cell in the presence of an anti-viral drug. This invention is also related to the means and methods of identifying anti-viral drugs which have distinct patterns of resistance when compared with existing anti-viral drugs. This invention also relates to methods and compositions for the identification and assessment of the biological effectiveness of potential therapeutic compounds. This invention is more particularly related to drug susceptibility and resistance tests useful in providing an optimal therapeutic regimen for the treatment of various viral diseases, including for example, HIV/AIDS and hepatitis.

-2-

Background of the Invention

Viral Drug Resistance

The use of anti-viral compounds for chemotherapy and chemoprophylaxis of viral diseases is a relatively new development in the field of infectious diseases, particularly when compared with the more than 50 years of experience with antibacterial antibiotics. The design of anti-viral compounds is not straightforward because viruses present a number of unique problems. Viruses must replicate intracellularly and often employ host cell enzymes, macromolecules, and organelles for the synthesis of virus particles. Therefore, safe and effective anti-viral compounds must be able to discriminate with a high degree of efficiency between cellular and virus-specific functions. In addition, because of the nature of virus replication, evaluation of the *in vitro* sensitivity of virus isolates to anti-viral compounds must be carried out in a complex culture system consisting of living cells (e.g. tissue culture). The results from such assay systems vary widely according to the type of tissue culture cells which are employed and the conditions of assay. Despite these complexities nine drugs have been approved for AIDS therapy, five reverse transcriptase inhibitors AZT, ddI, ddC, d4T, 3TC, one non-nucleoside reverse transcriptase inhibitor, nevirapine and three protease inhibitors saquinavir, ritonavir and indinovir and several additional anti-viral drug candidates have been recently developed such as nelfinavir, delaviridine, VX-478 and 1592.

Viral drug resistance is a substantial problem given the high rate of viral replication and mutation frequencies. Drug resistant mutants were first recognized for poxviruses with thiosemicarbazone (Appleyard and Way (1966) *Brit. J. Exptl. Pathol.* 47, 144-51), for poliovirus with guanidine

(Melnick et al. (1961) *Science* **134**, 557), for influenza A
virus with amantadine (Oxford et al. (1970) *Nature* **226**,
82-83; Cochran et al. (1965) *Ann. NY Acad Sci* **130**, 423-429)
and for herpes simplex virus with iododeoxyuridine (Jawetz
et al. (1970) *Ann. NY Acad Sci* **173**, 282-291). Approximately
75 HIV drug resistance mutations to various anti-viral
agents have been identified to date (Mellors et al. (1995)
Intnl. Antiviral News, supplement and Condra, J.H. et al.
(1996) *J Virol.* **70**, 8270-8276).

The small and efficient genomes of viruses have lent
themselves to the intensive investigation of the molecular
genetics, structure and replicative cycles of most important
human viral pathogens. As a consequence, the sites and
mechanisms have been characterized for both the activity of
and resistance to anti-viral drugs more precisely than have
those for any other class of drugs. (Richman (1994) *Trends
Microbiol.* **2**, 401-407). The likelihood that resistant
mutants will emerge is a function of at least four factors:
1) the viral mutation frequency; 2) the intrinsic mutability
of the viral target site with respect to a specific
anti-viral; 3) the selective pressure of the anti-viral
drug; and, 4) the magnitude and rate of virus replication.
With regard to the first factor, for single stranded RNA
viruses, whose genome replication lacks a proofreading
mechanism, the mutation frequencies are approximately 3×10^{-5}
per base-pair per replicative cycle (Holland et al. (1992)
Curr. Topics Microbiol Immunol. **176**, 1-20; Mansky et al.
(1995) *J Virol.* **69**, 5087-94; Coffin (1995) *Science* **267**,
483-489). Thus, a single 10 kilobase genome, like that of
human immunodeficiency virus (HIV), would be expected to
contain on average one mutation for every three progeny
viral genomes. As to the second factor, the intrinsic
mutability of the viral target site in response to a
specific anti-viral agent can significantly affect the

likelihood of resistant mutants. For example, zidovudine (AZT) selects for mutations in the reverse transcriptase of HIV more readily *in vitro* and *in vivo* than does the other approved thymidine analog d4T (stavudine).

One, perhaps inevitable consequence of the action of an anti-viral drug is that it confers sufficient selective pressure on virus replication to select for drug-resistant mutants (Herrmann et al. (1977) *Ann NY Acad Sci* **284**, 632-7). With respect to the third factor, with increasing drug exposure, the selective pressure on the replicating virus population increases to promote the more rapid emergence of drug resistant mutants. For example, higher doses of AZT tend to select for drug resistant virus more rapidly than do lower doses (Richman et al. (1990) *J. AIDS*. **3**, 743-6). This selective pressure for resistant mutants increases the likelihood of such mutants arising as long as significant levels of virus replication are sustained.

The fourth factor, the magnitude and rate of replication of the virus population, has major consequences on the likelihood of emergence of resistant mutants. Many virus infections are characterized by high levels of virus replication with high rates of virus turnover. This is especially true of chronic infections with HIV as well as hepatitis B and C viruses. The likelihood of emergence of AZT resistance increases in HIV-infected patients with diminishing CD4 lymphocyte counts which are associated with increasing levels of HIV replication (Ibid).

Higher levels of virus increase the probability of preexisting mutants. It has been shown that the emergence of a resistant population results from the survival and selective proliferation of a previously existing subpopulation that randomly emerges in the absence of

selective pressure. All viruses have a baseline mutation rate. With calculations of approximately 10^{10} new virions being generated daily during HIV infection (Ho et al. (1995) *Nature* 373, 123-126), a mutation rate of 10^{-4} to 10^{-5} per nucleotide guarantees the preexistence of almost any mutation at any time point during HIV infection. Evidence is accumulating that drug resistant mutants do in fact exist in subpopulations of HIV infected individuals (Najera et al. (1994) *AIDS Res Hum Retroviruses* 10, 1479-88; Najera et al. (1995) *J Virol.* 69, 23-31). The preexistence of drug resistant picornavirus mutants at a rate of approximately 10^{-5} is also well documented (Ahmad et al. (1987) *Antiviral Res.* 8, 27-39).

Human Immunodeficiency Virus (HIV)

Acquired immune deficiency syndrome (AIDS) is a fatal human disease, generally considered to be one of the more serious diseases to ever affect humankind. Globally, the numbers of human immunodeficiency virus (HIV) infected individuals and of AIDS cases increase relentlessly and efforts to curb the course of the pandemic, some believe, are of limited effectiveness. Two types of HIV are now recognized: HIV-1 and HIV-2. By December 31, 1994 a total of 1,025,073 AIDS cases had been reported to the World Health Organization. This is only a portion of the total cases, and WHO estimates that as of late 1994, allowing for underdiagnosis, underreporting and delays in reporting, and based on the estimated number of HIV infections, there have been over 4.5 million cumulative AIDS cases worldwide (Mertens et al. (1995) *AIDS* 9 (Suppl A), S259-S272). Since HIV began its spread in North America, Europe and sub-Saharan Africa, over 19.5 million men, women and children are estimated to have been infected (Ibid). One of the distinguishing features of the AIDS pandemic has been its global spread within the last 20 years, with about 190 countries reporting AIDS cases

today. The projections of HIV infection worldwide by the WHO are staggering. The projected cumulative total of adult AIDS cases by the year 2000 is nearly 10 million. By the year 2000, the cumulative number of HIV-related deaths in adults is predicted to rise to more than 8 million from the current total of around 3 million.

HIV-1 and HIV-2 are enveloped retroviruses with a diploid genome having two identical RNA molecules. The molecular organization of HIV is (5') U3-R-U5-gag-pol-env-U3-R-U5 (3') as shown in Fig. 1a. The U3, R, and U5 sequences form the long terminal repeats (LTR) which are the regulatory elements that promote the expression of the viral genes and sometimes nearby cellular genes in infected hosts. The internal regions of the viral RNA code for the structural proteins: gag (p55, p17, p24 and p7 core proteins), pol (p10 protease, p66 and p51 reverse transcriptase and p32 integrase) and env (gp120 and gp41 envelope glycoproteins). Gag codes for a polyprotein precursor that is cleaved by a viral protease into three or four structural proteins; pol codes for reverse transcriptase (RT) and the viral protease and integrase; env codes for the transmembrane and outer glycoprotein of the virus. The gag and pol genes are expressed as a genomic RNA while the env gene is expressed as a spliced subgenomic RNA. In addition to the env gene there are other HIV genes produced by spliced subgenomic RNAs that contribute to the replication and biologic activities of the virus. These genes include: tat which encodes a protein that activates the expression of viral and some cellular genes; rev which encodes a protein that promotes the expression of unspliced or single-spliced viral mRNAs; nef which encodes a myristylated protein that appears to modulate viral production under certain conditions; vif which encodes a protein that affects the ability of virus particles to infect target cells but does not appear to

affect viral expression or transmission by cell-to-cell contact; *vpr* which encodes a virion-associated protein; and *vpu* which encodes a protein that appears to promote the extracellular release of viral particles.

5

No disease better exemplifies the problem of viral drug resistance than AIDS. Drug resistant HIV isolates have been identified for nucleoside and non-nucleoside reverse transcriptase inhibitors and for protease inhibitors. The emergence of HIV isolates resistant to AZT is not surprising since AZT and other reverse transcriptase inhibitors only reduce virus replication by about 90%. High rates of virus replication in the presence of the selective pressure of drug treatment provide ideal conditions for the emergence of drug-resistant mutants. Patients at later stages of infection who have higher levels of virus replication develop resistant virus with AZT treatment more quickly than those at early stages of infection (Richman et al. (1990) *J AIDS* 3, 743-6). The initial description of the emergence of resistance to AZT identified progressive and stepwise reductions in drug susceptibility (Larder et al. (1989) *Science* 243, 1731-1734). This was explained by the recognition of multiple mutations in the gene for reverse transcriptase that contributed to reduced susceptibility (Larder et al. (1989) *Science* 246, 1155-1158). These mutations had an additive or even synergistic contribution to the phenotype of reduced susceptibility (Kellam et al. (1992) *Proc. Natl. Acad. Sci.* 89, 1934-1938). The cumulative acquisition of such mutations resulted in progressive decreases in susceptibility. Similar effects have been seen with non-nucleoside reverse transcriptase inhibitors (Nunberg et al. (1991) *J Virol* 65, 4887-4892; Sardanna et al. (1992) *J Biol Chem* 267, 17526-17530). Studies of protease inhibitors have found that the selection of HIV strains with reduced drug susceptibility occurs

within weeks (Ho et al. (1994) *J Virol* **68**, 2016-2020; Kaplan et al. (1994) *Proc. Natl. Acad. Sci.* **91**, 5597-5601). While recent studies have shown protease inhibitors to be more powerful than reverse transcriptase inhibitors, nevertheless resistance has developed. (Condra et al., Id. and Report 3rd Conference on Retroviruses and Opportunistic Infections, March 1996). Subtherapeutic drug levels, whether caused by reduced dosing, drug interactions, malabsorption or reduced bioavailability due to other factors, or self-imposed drug holidays, all permit increased viral replication and increased opportunity for mutation and resistance. (Id.)

The selective pressure of drug treatment permits the outgrowth of preexisting mutants. With continuing viral replication in the absence of completely suppressive anti-viral drug activity, the cumulative acquisition of multiple mutations can occur over time, as has been described for AZT and protease inhibitors of HIV. Indeed viral mutants multiply resistant to different drugs have been observed (Larder et al. (1989) *Science* **243**, 1731-1734; Larder et al. (1989) *Science* **246**, 1155-1158; Condra et al. (1995) *Nature* **374**, 569-71). With the inevitable emergence of resistance in many viral infections, as with HIV for example, strategies must be designed to optimize treatment in the face of resistant virus populations. Ascertaining the contribution of drug resistance to drug failure is a difficult problem because patients who are more likely to develop drug resistance are more likely to have other confounding factors that will predispose them to a poor prognosis (Richman (1994) *AIDS Res Hum Retroviruses* **10**, 901-905). In addition patients contain mixtures of viruses with different susceptibilities.

Hepatitis B (HBV)

HBV is a causative agent for acute and chronic hepatitis, which strikes about 200 million patients worldwide. Zuckerman A.J. *Trans. R. Soc. Trop. Med. Hygiene* (1982) **76**, 711-718. HBV infection acquired in adult life is often clinically inapparent, and most acutely infected adults recover completely from the disease and clear the virus. Rarely, however, the acute liver disease may be so severe that the patient dies of fulminant hepatitis. A small fraction, perhaps 5 to 10%, of acutely infected adults, becomes persistently infected by the virus and develops chronic liver disease of varying severity. Neonatally transmitted HBV infection, however, is rarely cleared, and more than 90% of such children become chronically infected. Because HBV is commonly spread from infected mother to newborn infant in highly populated areas of Africa and Asia, several hundred million people throughout the world are persistently infected by HBV for most of their lives and suffer varying degrees of chronic liver disease, which greatly increases their risk of developing cirrhosis and hepatocellular carcinoma (HCC). Indeed, the risk of HCC is increased 100-fold in patients with chronic hepatitis, and the lifetime risk of HCC in males infected at birth approaches 40%. Beasley RP et al., *Lancet* (1981) **2**, 1129-1133. Accordingly, a large fraction of the world's population suffers from and dies of these late complications of HBV infection. The development of anti-HBV drugs has been long awaited, but has been hampered by the extremely narrow host range of HBV: HBV replicates mainly in human and chimpanzee livers and not in experimental animals or in cultured cells. Tiollais, P et al. *Nature* (London) (1985) **317**, 489-495.

-10-

Hepatitis B virus is a DNA virus with a compact genomic structure; despite its small, circular, 3200 base pairs, HBV DNA codes for four sets of viral products and has a complex, multiparticle structure. HBV achieves its genomic economy
5   by relying on an efficient strategy of encoding proteins from four overlapping genes: S, C, P, and X. HBV is one of a family of animal viruses, hepadnaviruses, and is classified as hepadnavirus type 1. Similar viruses infect certain species of woodchucks, ground and tree squirrels,
10 and Pekin ducks. All hepadnaviruses, including HBV, share the following characteristics: 1) three distinctive morphological forms exist, 2) all members have proteins that are functional and structural counterparts to the envelope and nucleocapsid antigens of HBV, 3) they replicate within
15 the liver but can also exist in extrahepatic sites, 4) they contain an endogenous DNA polymerase with both RNA- and DNA-dependent DNA polymerase activities, 5) their genomes are partially double stranded circular DNA molecules, 6) they are associated with acute and chronic hepatitis and
20 hepatocellular carcinoma and 7) replication of their genome goes through an RNA intermediate which is reverse transcribed into DNA using the virus's endogenous RNA-dependent DNA polymerase activity in a manner analogous to that seen in retroviruses. In the nucleus of infected
25 liver cells, the partially double stranded DNA is converted to a covalently closed circular double stranded DNA (cccDNA) by the DNA-dependent DNA polymerase. Transcription of the viral DNA is accomplished by a host RNA polymerase and gives rise to several RNA transcripts that differ in their
30 initiation sites but all terminate at a common polyadenylation signal. The longest of these RNAs acts as the pregenome for the virus as well as the message for the some of the viral proteins. Viral proteins are translated from the pregenomic RNAs, and the proteins and RNA pregenome
35 are packaged into virions and secreted from the hepatocyte.

Although HBV is difficult to cultivate in vitro, several
cells have been successfully transfected with HBV DNA
resulting in the <u>in vitro</u> production of HBV particles.

5   There are three particulate forms of HBV: non-infectious
22nm particles, which appear as either spherical or long
filamentous forms, and 42nm double-shelled spherical
particles which represent the intact infectious hepatitis B
virion. The envelope protein, HBsAg, is the product of the
10   S gene of HBV and is found on the outer surface of the
virion and on the smaller spherical and tubular structures.
Upstream of the S gene open reading frame are the pre-S gene
open reading frames, pre-S1 and pre-S2, which code for the
pre-S gene products, including receptors on the HBV surface
15   for polymerized human serum albumin and the attachment sites
for hepatocyte receptors. The intact 42nm virion can be
disrupted by mild detergents and the 27nm nucleocapsid core
particle isolated. The core is composed of two nucleocapsid
proteins coded for by the C gene. The C gene has two
20   initiation codons defining a core and a precore region. The
major antigen expressed on the surface of the nucleocapsid
core is coded for by the core region and is referred to as
hepatitis B core antigen (HBcAg). Hepatitis B e antigen
(HBeAg) is produced from the same C gene by initiation at
25   the precore ATG.

Also packaged within the nucleocapsid core is a DNA
polymerase, which directs replication and repair of HBV DNA.
The DNA polymerase is coded for by the P gene, the third and
30   largest of the HBV genes. The enzyme has both DNA-dependent
DNA polymerase and RNA-dependent reverse transcriptase
activities and is also required for efficient encapsidation
of the pregenomic RNA. The fourth gene, X, codes for a
small, non-particle-associated protein which has been shown
35   to be capable of transactivating the transcription of both

viral and cellular genes.

Although HBV replication is fairly well understood, early
steps in HBV infection have not been well defined. Cellular
5   receptors or attachment sites on the virions cannot be
studied without appropriate tissue culture assays. In an
effort to address this problem, certain cell lines have been
developed, human hepatoblastoma cells Huh (HB 611) (Ueda, K.
et al., *Virology* (1989) **169**, 213-216) and HepG2 cells
10   (Galle, P.R. and Theilmann, L. *Arzneim-Forsch. Drug Res.*
(1990) **40**, 1380-1382) for evaluation of anti-HBV drugs.

Recently, attention has focused on molecular variants of
HBV. Variation occurs throughout the HBV genome, and
15   clinical isolates of HBV that do not express viral proteins
have been attributed to mutation in individual or even
multiple gene locations. For example, variants have been
described which lack nucleocapsid proteins, envelope
proteins, or both. Two mutants have attracted attention.
20   The first is found in certain patients with severe chronic
HBV infection. These patients were found to be infected
with an HBV mutant that contained an alteration in the
precore region rendering the virus incapable of encoding
HBeAg. The most commonly encountered mutation in such
25   patients is a single base substitution, from G to A, which
occurs in the second to last codon of the pre-C gene at
nucleotide 1896. This substitution results in the
replacement of the TGG tryptophan codon by a stop codon
(TAG), which prevents the translation of HBeAg. Patients
30   with such precore mutants that are unable to secrete HBeAg
tend to have severe liver disease that progresses rapidly to
cirrhosis and that does not respond readily to anti-viral
therapy.

35

The second category of HBV mutants consists of escape mutants, in which a single amino acid substitution, from glycine to arginine, occurs at position 145 of the immunodominant a determinant common to all subtypes of
5   HBsAg. This change in HBsAg leads to a critical conformational change that results in a loss of neutralizing activity by anti-HBs antibody.

Presently Available Viral Resistance Assays
10 The definition of viral drug susceptibility is generally understood to be the concentration of the anti-viral agent at which a given percentage of viral replication is inhibited (e.g. the IC_{50} for an anti-viral agent is the concentration at which 50% of virus replication is
15 inhibited). Thus, a decrease in viral drug susceptibility is the hallmark that an anti-viral has selected for mutant virus that is resistant to that anti-viral drug. Viral drug resistance is generally defined as a decrease in viral drug susceptibility in a given patient over time. In the
20 clinical context, viral drug resistance is evidenced by the anti-viral drug being less effective or no longer being clinically effective in a patient.

At present the tools available to the researcher and
25 clinician to assess anti-viral drug susceptibility and resistance are inadequate. The classical test for determining the resistance and sensitivity of HIV to an anti-viral agent is complex, time-consuming, expensive, and is hazardous in that it requires the culture of pathogenic
30 virus from each and every patient (Barre-Sinoussi et al (1983) *Science* 220, 868-871; Popovic et al. (1984) *Science* 224, 497-500). In this procedure, the patient's peripheral blood mononuclear cells (PBMC) are first cultured to establish a viral stock of known multiplicity of infection
35 (moi), and the viral stock thereby produced is used to

-14-

infect a target indicator cell line. The resulting burst of viral replication is then typically measured in the presence and absence of an anti-viral agent by determining the production of viral antigens in the cell culture. Such tests can be performed reliably only in the hands of expert investigators, and may take two to three months to carry out at a cost of thousands of dollars per patient for each agent tested. Furthermore, as viral stocks of sufficient moi cannot be established from the PBMC of some HIV patients, the classical test for HIV resistance cannot be performed on all HIV-infected individuals. More significantly, in the course of generating the viral stock by passage of the virus in culture, the characteristics of the viruses themselves can change and may therefore obscure the true nature of the patient's virus. Thus, the application of the classical test has been limited to gathering information about trends in clinical trials and has not been available for a prospective analysis which could be used to custom tailor anti-viral therapy for a given patient. Notwithstanding these limitations, the classical test has two important qualities: it is *specific* for the agent under evaluation, and it provides information on the *phenotype* of the patient's own virus, that is, the concentration of the drug which inhibits 50% of viral replication (IC_{50}).

A number of attempts have been made to improve upon the classical test, but each of these has serious shortcomings. The first type of these tests can be described as *nonspecific* in that they do not determine the characteristics of a patient's own virus at all, but rather provide an independent measure of the course of the infection. Among these tests are those which measure the patient's CD4+ T cell count, the hallmark of HIV disease progression (Goedert et al. (1987) *JAMA* 257, 331-334), those which measure viral antigen levels (e.g., p24 core antigen

(Allain et al. (1987) *N. Engl. J. Med.* 317, 1114-1121)), and
those which measure viral RNA and DNA levels (e.g.,
quantitative polymerase chain reaction and branched DNA
assays (Piatak et al. (1993) *Science* 259, 1749-1754; Urdea
(1993) *Clin. Chem.* 39, 725-726)). The primary disadvantage
of such nonspecific tests is that they do not provide any
information on viral drug resistance per se, but rather
attempt to infer this information from the apparent course
of the patient's disease. In addition, many factors other
than viral drug resistance can affect the level of the
parameter under consideration. In other words, CD4⁺ T cell
counts, p24 antigen levels and HIV viral RNA levels can vary
for reasons other than drug resistance during the course of
disease.

Another modified classical test amplifies the viral gene
that is the target of the anti-viral agent. In this test
the viral gene from a given patient is amplified and then
recombined into a biologically active proviral clone of HIV.
This proviral clone is transfected into human cells to
generate a viral stock of known moi which can then be used
to infect a target indicator cell line. In the manner of
the classical test, one then determines the production of
viral antigens in the presence or absence of the anti-viral
agent. One such assay described by Kellam and Larder (1994)
Antimicrobial Agents and Chemo. 38, 23-30, involves PCR
amplification of reverse transcriptase coding sequences from
a patient, which is then introduced into a proviral DNA
clone by homologous recombination to reconstitute the
complete viral genome including the reverse transcriptase
gene which was deleted. The resulting recombinant virus
produced from such clones is then cultured in T-cell lines,
and the drug sensitivity is tested in the HeLa CD4⁺ plaque
reduction assay. However, this class of test still requires
the culturing of virus to determine drug resistance, and is

thus difficult, lengthy and costly and requires the laboratory investigator to handle hazardous viral cultures. Furthermore, given the attendant variation of the virus itself during the culture process, the results may be correspondingly inaccurate.

A second class of test attempts to provide specific information on the genotype of the patient's HIV, with the ultimate goal of correlating this genotypic information with the virus' drug resistant phenotype. Indeed, specific amino acid substitutions within viral genes such as reverse transcriptase and protease genes have been shown to correspond to specific levels of viral resistance to reverse transcriptase and protease inhibitors, respectively (Larder et al. (1994) *J. Gen Virol.* 75, 951-957). A major shortcoming associated with such an analysis is that it is indirect and can be obfuscated by secondary mutations which have been shown to add to or counter the effects of the first mutation. It is the complex interplay of all amino acid residues within a given viral polypeptide which ultimately determines the gene product's activity in the presence or absence of an inhibitor. Thus, a database of vast and impractical proportions would be necessary to interpret the status of drug resistance or sensitivity of a given genotype, given the number of potential amino acid changes in the HIV genome.

A third class of test, a recently developed bacterial-based assay makes use of a molecularly cloned viral gene (specifically, the reverse transcriptase gene) which has been inserted into a bacterial expression vector. Upon transformation of special strains of *E. coli* which are deficient in the bacterial DNA polymerase I, the cloned reverse transcriptase gene can rescue the growth of the bacteria under selected growth conditions. In making *E.*

coli dependent upon reverse transcriptase for their growth, one can ascertain the effects of certain reverse transcriptase inhibitors on the activity of the viral gene (PCT Application No. WO 95/22622). A major shortcoming with this approach, however, is that the inhibitor may be transported across the cell membrane and metabolized differently by the bacteria than it is by a human cell, and as a result the concentration of the true metabolic inhibitor of the reverse transcriptase may be grossly different in the bacterium than it would be in a relevant human cell target of infection, or the true inhibitor may be absent altogether. Indeed, nucleoside metabolism is known to differ markedly between human and bacterial cells. Another significant shortcoming of this approach is that the assay measures DNA-dependent DNA polymerase activity of reverse transcriptase but not the RNA-dependent DNA polymerase, strand transfer or RNAse H activities of the reverse transcriptase. Thus, an anti-viral compound which acts, at least in part, on these other activities would not have its full inhibitory activity in this assay. Yet another difficulty with this approach is that it is a growth-based test; thus if an inhibitor (eg., a nucleoside analog) also blocks bacterial growth for reasons other than its effects on reverse transcriptase, it can not be adequately tested in this system.

Viral Vectors

Viral vectors and particularly retroviral vectors have been used for modifying mammalian cells because of the high efficiency with which retroviral vectors infect target cells and integrate into the target cell genome. Because of their ability to insert into the genome of mammalian cells much attention has focused on retroviral vectors for use in gene therapy. Details on retroviral vectors and their use can be found in patents and patent publications including European

-18-

Patent Application EPA 0 178 220, U.S. Patent 4,405,712, PCT
Application WO 92/07943, U.S. Patent 4,980,289, U.S. Patent
5,439,809 and PCT Application WO 89/11539. The teachings
of these patents and publications are incorporated herein by
5   reference.

One consequence of the emphasis on retroviral vector
technology has been the development of packaging cell lines.
A major problem with the use of retroviruses is the
10   possibility of the spread of replication-competent
retrovirus. There is thus a need for producing helper
vectors which could not be processed into virions. As a
result packaging defective vectors and packaging cell lines
were developed. Details on packaging-defective vectors and
15   packaging cell lines can be found in patents and patent
publications, including U.S. Patent 5,124,263, European
Patent Application Pub. No. 0386 882, PCT Application No. WO
91/19798, PCT Application No. WO 88/08454, PCT Application
No. WO 93/03143, U.S. Patent No. 4,650,764, U.S. Patent
20   4,861,719 and U.S. Patent 5,278,056, the disclosures of
which are incorporated herein by reference.

It is an object of this invention to provide a drug
susceptibility and resistance test capable of showing
25   whether a viral population in a patient is resistant to a
given prescribed drug. Another object of this invention is
to provide a test that will enable the physician to
substitute one or more drugs in a therapeutic regimen for a
patient that has become resistant to a given drug or drugs
30   after a previous course of therapy. Yet another object of
this invention is to provide a test that will enable
selection of an effective drug regimen for the treatment of
virus infections. Yet another object of this invention is
to provide a safe, standardized, affordable, rapid, precise
35   and reliable assay of drug susceptibility and resistance for

-19-

clinical and research application. Still another object of
this invention is to provide a test and methods for
evaluating the biological effectiveness of candidate drug
compounds which act on specific viral genes and/or viral
5   proteins particularly with respect to viral drug resistance
and cross resistance.   It is also an object of this
invention to provide the means and compositions for
evaluating viral drug resistance and susceptibility. This
and other objects of this invention will be apparent from
10.   the specification as a whole.

Summary of the Invention

Objects of the present invention are accomplished by a novel test for determining susceptibility for an anti-viral drug comprising: (a) introducing a resistance test vector, comprising a patient-derived segment and an indicator gene into a host cell; (b) culturing the host cell from step (a); (c) measuring expression of the indicator gene in a target host cell; and (d) comparing the expression of the indicator gene from step (c) with the expression of the indicator gene measured when steps (a)-(c) are carried out in the absence of the anti-viral drug, wherein a test concentration of the anti-viral drug is present at steps (a)-(c); at steps (b)-(c); or at step (c).

This invention also provides a method for determining anti-viral drug resistance in a patient comprising: (a) developing a standard curve of drug susceptibility for an anti-viral drug; (b) determining anti-viral drug susceptibility in the patient using the susceptibility test described above; and (c) comparing the anti-viral drug susceptibility in step (b) with the standard curve determined in step (a), wherein a decrease in anti-viral susceptibility indicates development of anti-viral drug resistance in the patient.

This invention further provides a method for determining anti-viral drug resistance in a patient comprising: (a) determining anti-viral drug susceptibility in the patient at a first time according to the above method, wherein the patient-derived segment is obtained from the patient at said time; (b) determining anti-viral drug susceptibility of the same patient at a later time; and (c) comparing the anti-viral drug susceptibilities determined in step (a) and (b), wherein a decrease in anti-viral drug susceptibility at

-21-

the later time compared to the first time indicates the
development or progression of anti-viral drug resistance in
the patient.

5   The assay of this invention enables a physician to assess
       whether a viral gene encoding a viral protein or a
       functional viral sequence, each of which may be the target
       of an anti-viral agent, has mutated to render the drug less
       effective. More particularly, the novel assay of this
10   invention enables one to determine whether a virus has
       become resistant to a particular anti-viral drug.
       Furthermore, this invention enables a physician to assess
       drug susceptibility and resistance of combination therapy.
       In addition the assay enables one to alter a therapeutic
15   regimen prospectively by testing particular drug(s) or
       combinations of drugs and determining whether these drugs,
       alone or in combination, inhibit one or more viral gene(s)
       and/or viral protein(s). This invention provides
       significant advantages over presently available assays by
20   providing a safer, more affordable, more reliable, more
       rapid and more effective drug susceptibility and resistance
       assay to assess the therapeutic efficacy of particular
       anti-viral drug(s) or combinations of drugs enabling a
       physician to optimize treatment. The assay of this
25   invention has the significant advantage of enabling the
       evaluation of resistance and susceptibility at all stages of
       drug development: 1) during preclinical evaluation of
       candidate compounds; 2) during clinical evaluation of new
       drugs; 3) during patient therapy enabling design of an
30   effective therapeutic regimen to overcome the problem of
       drug resistance; and 4) as part of epidemiologic
       surveillance, evaluating the prevalence of resistance during
       the use of approved and experimental drugs.

35   The present invention is directed to the methods and

compositions of assessing drug susceptibility and resistance, including: a) the assay method of determining the susceptibility and resistance of a patient-derived segment to an anti-viral drug; b) compositions including resistance test vectors comprising a patient-derived segment and an indicator gene; and c) host cells containing the resistance test vectors. This invention is further directed to the compositions and methods of constructing the vectors and host cells which are used in the drug susceptibility and resistance assay of this invention.

In one aspect of the invention there is provided a method for determining susceptibility for an anti-HIV drug comprising: (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell; (b) culturing the host cell from step (a); (c) measuring expression of the indicator gene in a target host cell; and (d) comparing the expression of the indicator gene from step (c) with the expression of the indicator gene measured when steps (a)-(c) are carried out in the absence of the anti-HIV drug, wherein a test concentration of the anti-HIV drug is present at steps (a)-(c); at steps (b)-(c); or at step (c).

In one aspect of the invention there is provided a method for determining susceptibility for an anti-HIV drug comprising: (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell; (b) culturing the host cell from step (a); (c) measuring an indicator in a target host cell wherein said indicator is a DNA or RNA structure; and (d) comparing the measurement of the indicator from step (c) with the measurement of the indicator when steps (a)-(c) are carried

-23-

out in the absence of the anti-HIV drug, wherein a test
concentration of the anti-HIV drug is present at steps
(a)-(c); at steps (b)-(c); or at step (c).

5   In one aspect of the invention there is provided a method
for determining susceptibility for an anti-HBV drug
comprising: (a) introducing a resistance test vector
comprising a patient-derived segment and an indicator gene
into a host cell; (b) culturing the host cell from step (a);
10   (c) measuring expression of the indicator gene in a target
host cell; and (d) comparing the expression of the indicator
gene from step (c) with the expression of the indicator gene
measured when steps (a)-(c) are carried out in the absence
of the anti-HBV drug, wherein a test concentration of the
15   anti-HBV drug is present at steps (a)-(c); at steps (b)-(c);
or at step (c).

In one aspect of the invention there is provided a method
for determining susceptibility for an anti-HBV drug
20   comprising: (a) introducing a resistance test vector
comprising a patient-derived segment and an indicator gene
into a host cell; (b) culturing the host cell from step (a);
(c) measuring an indicator in a target host cell wherein
said indicator is a DNA or RNA structure; and (d) comparing
25   the measurement of the indicator from step (c) with the
measurement of the indicator when steps (a)-(c) are carried
out in the absence of the anti-HBV drug, wherein a test
concentration of the anti-HBV drug is present at steps
(a)-(c); at steps (b)-(c); or at step (c).
30
This invention also provides a method for determining
anti-HIV drug resistance in a patient comprising: (a)
developing a standard curve of drug susceptibility for an
anti-HIV drug; (b) determining anti-HIV drug susceptibility
35   in the patient using the susceptibility test described

-24-

above; and (c) comparing the anti-HIV drug susceptibility in step (b) with the standard curve determined in step (a), wherein a decrease in anti-HIV susceptibility indicates development of anti-HIV drug resistance in the patient.

5

This invention also provides a method for determining anti-HBV drug resistance in a patient comprising:(a) developing a standard curve of drug susceptibility for an anti-HBV drug; (b) determining anti-HBV drug susceptibility in the patient using the susceptibility test described above; and (c) comparing the anti-HBV drug susceptibility in step (b) with the standard curve determined in step (a), wherein a decrease in anti-HBV susceptibility indicates development of anti-HBV drug resistance in the patient.

10

15

This invention also provides a method for evaluating the biological effectiveness of a candidate anti-viral drug compound comprising: (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell; (b) culturing the host cell from step (a); (c) measuring expression of the indicator gene in a target host cell; and (d) comparing the expression of the indicator gene from step (c) with the expression of the indicator gene measured when steps (a)-(c) are carried out in the absence of the candidate anti-viral drug compound, wherein a test concentration of the candidate anti-viral drug compound is present at steps (a)-(c); at steps (b)-(c); or at step (c).

20

25

This invention also provides a method for evaluating the biological effectiveness of a candidate anti-viral drug compound comprising: (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell; (b) culturing the host cell from step (a); (c) measuring an indicator in a target host cell

30

35

-25-

wherein said indicator is a DNA or RNA structure; and (d)
comparing the measurement of the indicator from step (c)
with the measurement of the indicator when steps (a)-(c) are
carried out in the absence of the candidate anti-viral drug
5   compound, wherein a test concentration of the candidate
anti-viral drug compound is present at steps (a)-(c); at
steps (b)-(c); or at step (c).

The resistance test vector comprising the patient-derived
10   viral segment(s) (e.g. HIV, HBV, etc.) and the indicator
gene   may   additionally   include   one   or   more
non-patient-derived   segments.   In   one   embodiment   the
resistance test vector is constructed from a genomic viral
vector which may include a deletion in one or more genes.
15   For example, in the case of HIV, *env* is deleted in a
resistance test vector which otherwise preserves the mRNA
expression and processing characteristics of the complete
virus.   Alternatively,   the   resistance   test   vector   is
constructed from a subgenomic viral vector which may include
20   only one or a few viral genes which are typically the target
of the anti-viral drug.   For example in the case of HBV, one
or more of the HBV genes encoding certain structural and
enzymatic functions necessary for HBV DNA replication and
virus particle formation (i.e. C, S and X genes) may be
25   deleted from a resistance test vector which otherwise
preserves mRNA expression and processing characteristics of
the complete virus.   The resistance test vector further
comprises   either   the   native   enhancer/promoter   of   the
particular virus or a foreign enhancer/promoter for the
30   expression of the anti-viral target genes.

The expression of the indicator gene in the resistance test
vector in the target cell is ultimately dependent upon the
action of the patient-derived segment sequences.   The
35   indicator gene may be functional or non-functional.   In the

case of a non-functional indicator gene, the indicator gene is not efficiently expressed in a host cell transfected by the resistance test vector until it is converted into a functional indicator gene through the action of one or more of the patient-derived segment products. In one embodiment, the indicator gene is rendered non-functional through use of a permuted promoter, i.e. a promoter that, although in the same transcriptional orientation as the indicator gene, follows rather than precedes the coding sequence of the indicator gene. In addition, the orientation of the non-functional indicator gene is opposite to that of the viral promoters or LTRs. Thus the coding sequence of the non-functional indicator gene can neither be transcribed by the permuted promoter nor by the viral promoters. In the case of HIV, the indicator gene is rearranged as a consequence of reverse transcription so that the permuted promoter now precedes the indicator gene sequence, which as a result can be functionally expressed. In the case of HBV, the indicator gene is rearranged as a consequence of circularization of the genome during HBV replication. In a second embodiment, the indicator gene is rendered non-functional through use of a permuted coding region, i.e. an indicator gene coding region in which the 5' portion of the coding region follows rather than precedes the 3' portion of the coding region. In this configuration, no mRNA is expressed which can give rise to a functional indicator gene product. In the case of HIV, the indicator gene is rearranged as a consequence of reverse transcription so that the 5' coding region of the indicator gene now precedes the 3' coding region, and as a result the indicator gene can be functionally expressed. In the case of HBV, the indicator gene is rearranged as a result of circularization of the genome during HBV replication. In a third embodiment, the indicator gene is rendered non-functional through use of an inverted intron, i.e. an intron inserted

-27-

into the coding sequence of the indicator gene with a transcriptional orientation opposite to that of the indicator gene. In addition, the indicator gene itself contains a functional promoter with the entire

5   transcriptional unit oriented opposite to the viral promoters. Thus the non-functional indicator gene is in the wrong orientation to be transcribed by the viral LTRs in the case of HIV or the HBV enhancer-promoter and it cannot be functionally transcribed by its own promoter, as the

10   inverted intron cannot be properly excised by splicing. However, in the case of retroviruses and hepadnaviruses, and HIV and HBV specifically, transcription by the viral promoters result in the formation of mRNA in which removal of the inverted intron can occur by splicing. In

15   retroviruses, as a consequence of reverse transcription of the resulting spliced transcript and the integration of the resulting provirus into the host cell chromosome, the indicator gene can now be functionally transcribed by its own promoter. In HBV, as a consequence of reverse

20   transcription of the resulting spliced transcript and circularization of the genomic DNA in the host cell, the indicator gene can now be functionally transcribed by its own promoter.

25

Resistance test vectors comprising a non-functional indicator gene can be used to carry out resistance tests in either a particle-based or non-particle-based assay. The

30   particle based assay is based on resistance test vector viral particles, which are replication defective, being produced by the resistance test vector host cells. The trans-acting factors necessary for production of the resistance test vector viral particles are provided by the

35   packaging expression vectors which are transfected into the

packaging host cell. In contrast the non-particle based resistance test is performed by transfection of a single host cell with a resistance test vector in the absence of packaging expression vectors.

In the case of the functional indicator gene, the functional indicator gene is efficiently expressed in a first host cell transfected by the resistance test vector (referred to herein as a resistance test vector host cell). Thus, the function of the indicator gene in the resistance test vector host cell is not dependent on the patient-derived segment. However, the capacity of the indicator gene to be expressed in a second host cell (referred to herein as a target host cell) is dependent on the production of functional resistance test vector viral particles in the resistance test vector host cell. Thus, the activity of the indicator gene in the target host cells is dependent on the activity of the patient-derived segments.

In another aspect this invention is directed to anti-viral drug susceptibility and resistance tests for HIV/AIDS or HBV. Particular resistance test vectors of the invention for use in the HIV/AIDS anti-viral drug susceptibility and resistance test are identified as well as resistance test vector host cells. In yet another aspect this invention is directed to anti-viral drug susceptibility and resistance tests for hepatitis. Similarly in the case of HBV, particular resistance test vectors (also referred to herein as resistance test vector systems) of the invention for use in the HBV anti-viral drug susceptibility and resistance test are identified as well as resistance test vector host cells.

In yet another aspect this invention provides for the identification and assessment of the biological

effectiveness of potential therapeutic anti-viral compounds for the treatment of viral diseases. In still another aspect the invention is directed to a host cell transfected with one or more vectors to assess drug susceptibility. In another aspect, the invention is directed to a novel resistance test vector comprising a patient-derived viral gene(s) and an indicator gene.

Brief Description of the Drawings

Fig. 1. A.   Diagrammatic representation of the DNA genomic
structure of HIV-1.   Viral proteins are encoded
in each of the three reading frames by the *gag*,
pol, *vif*, *vpr*, *tat*, *rev*, *vpu*, *env* and *nef* genes.
The RNA is transcribed from viral DNA and
processed by viral and cellular enzymes, giving
rise to both genomic viral RNA and mRNA. The U3,
R and U5 elements of the viral long terminal
repeat (LTR) are indicated.

B.   Generalized diagrammatic representation of the
HIV genomic viral vector which contains the
following elements in a 5' to 3' orientation: 1)
an HIV-LTR U3 region, 2) an HIV-LTR R region, 3)
an HIV-LTR U5 region , 4) the coding regions of
the HIV *gag-pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, deleted
env, and *nef* genes, and 5) a 3' HIV-LTR.

C.   Generalized diagrammatic representation of the
HIV genomic viral vector which contains the
following elements in a 5' to 3' orientation: 1)
a CMV IE enhancer-promoter, 2) an HIV-LTR R
region, 3) an HIV-LTR U5 region, 4) the coding
regions of the HIV *gag-pol*, *vif*, *vpr*, *tat*, *rev*,
vpu, deleted *env*, and *nef* genes, and 5) a 3'
HIV-LTR.

D.   Generalized diagrammatic representation of the
HIV subgenomic viral vector which contains the
following elements in a 5' to 3' orientation: 1)
an HIV-LTR U3 region, 2) an HIV-LTR R region, 3)
an HIV-LTR U5 region, 4) the coding region of the
HIV *gag-pol* gene, and 5) a 3' HIV- LTR.

                                -31-

E.   Generalized diagrammatic representation of the
      HIV subgenomic viral vector which contains the
      following elements in a 5' to 3' orientation: 1)
 5   a CMV IE enhancer-promoter, 2) an HIV-LTR R
      region, 3) an HIV-LTR U5 region, 4) the coding
      region of the HIV *gag-pol* gene, and 5) a 3' HIV-
      LTR.

10 Fig. 2. A.   Diagrammatic representation of the DNA genomic
      structure of HIV-1.

B.   Diagrammatic representation of the resistance
      test vector comprising a nonfunctional indicator
15   gene comprising a permuted promoter having the
      following elements in a 5' to 3' orientation: 1)
      an HIV-LTR U3 region (pLG-lucPP-HS and
      pLG-lucPP-PB) or a CMV IE enhancer-promoter
      (pCG-lucPP-HS and pCG-lucPP-PB), 2) an HIV-LTR R
20   region, 3) an HIV-LTR U5 region containing an
      inserted T7 phage RNA polymerase promoter (herein
      referred   to   as   T7   promoter)   with   a
      transcriptional orientation opposite to that of
      the LTRs, 4) the coding regions of the HIV
25   *gag-pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, deleted *env*,
      and *nef* genes, 5) a patient-derived segment(s)
      inserted into the patient sequence acceptor
      sites, 6) an indicator gene cassette inserted
      into the deleted *env* gene, and 7) a 3' HIV-LTR.

30

C.   Diagrammatic   representation   showing   the
      conversion of the non-functional indicator gene
      (permuted promoter) in the resistance test
      vector, described in 2(a), to a functional
35   indicator gene by reverse transcriptase. The

-32-

conversion to a functional indicator gene results from the repositioning of the T7 promoter relative to the indicator gene coding region.

5   Fig. 3. A.   Diagrammatic representation of the DNA genomic structure of HIV-1.

B.   Diagrammatic representation of the packaging expression vector pLTR-HIV3' which provides the vif, vpr, tat, rev, vpu and nef genes, each of which is expressed as a spliced subgenomic mRNA transcribed from the HIV LTR U3 region.

C.   Diagrammatic representation of the packaging expression vector pCMV-HIV3' which provides the vif, vpr, tat, rev, vpu and nef genes, each of which is expressed as a spliced subgenomic mRNA transcribed from the CMV IE enhancer-promoter.

D.   Diagrammatic representation of the packaging expression vector pVL-env4070A [pCXAS(4070A env)] which provides the amphotrophic MLV env gene product, by transcription from the CMV IE enhancer-promoter.

Fig. 4. A.   Diagrammatic representation of the DNA genomic structure of HIV-1.

B.   A generalized diagrammatic representation of the resistance test vectors comprising a nonfunctional indicator gene comprising a permuted coding region containing the following elements in a 5' to 3' orientation: 1) an HIV-LTR U3 region (pLG-lucPC-HS and pLG-lucPC-PB) or a first CMV IE enhancer-promoter (pCG-lucPC-HS

and pCG-lucPC-PB), 2) the HIV-LTR R and U5
regions, 3) the coding regions of the HIV
gag-*pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, deleted *env*,
and *nef* genes, 4) a patient-derived segment(s)
inserted into the patient sequence acceptor
sites, 5) a first indicator gene cassette
containing the 5' coding region of the luciferase
gene, inserted into the deleted *env* gene, 6) a
second indicator gene cassette containing the 3'
coding region of the luciferase gene, inserted
into a deleted 3' HIV-LTR U3 region, and 7) a 3'
HIV-LTR R and U5 region.

C.   Diagrammatic representation showing the
conversion of the non-functional indicator gene
(permuted coding region) in the resistance test
vector, described in 4(a), to a functional
indicator gene by reverse transcriptase.
Following reverse transcription and strand
transfer, the luciferase 3' coding region is
copied from the 3' LTR to the 5' LTR, permitting
the transcription of mRNA which can be spliced to
generate a functional luciferase coding region.

Fig. 5. A.   Diagrammatic representation of the DNA genomic
structure of HIV-1.

B.   A generalized diagrammatic representation of
the resistance test vectors comprising a
nonfunctional indicator gene comprising an
inverted intron containing the following elements
in a 5' to 3' orientation: 1) an HIV-LTR U3
region (pLG-lucII-HS and pLG-lucII-PB) or a first
CMV IE enhancer-promoter (pCG-lucII-HS and
pCG-lucII-PB), 2) the HIV-LTR R and U5 regions,

-34-

3) the coding regions of the HIV gag-*pol*, *vif*,
vpr, *tat*, *rev*, *vpu*, deleted *env*, and *nef* genes,
4) patient-derived segment(s) inserted into the
patient sequence acceptor site, 5) an indicator
gene cassette inserted into the deleted *env* gene,
and 5) a 3' HIV-LTR.

C.   Diagrammatic   representation   showing   the
conversion of the non-functional indicator gene
(inverted intron) in the resistance test vector,
described in 5(a), to a functional indicator gene
by   reverse   transcriptase.   The   overall
transcriptional orientation of the indicator gene
cassette is opposite to that of the first CMV
enhancer-promoter and viral LTRs, while the
orientation of the artificial intron is the same
as the latter elements. Transcription of the
indicator   gene   by   the   second   CMV
enhancer-promoter does not lead to the production
of functional transcripts as the inverted intron
cannot   be   spliced   in   this   orientation.
Transcription of the indicator gene by the 5'
viral LTR or the first CMV IE enhancer-promoter,
however, leads to the removal of the inverted
intron by RNA splicing, although the indicator
gene is still not functionally expressed as the
resulting   transcript   has   an   antisense
orientation. Following the reverse transcription
of this transcript and integration of the
resultant proviral DNA, the indicator gene can be
functionally transcribed by the second CMV
enhancer-promoter as the inverted intron has been
previously removed.

-35-

Fig. 6. A.   Diagrammatic representation of the DNA genomic
              structure of HIV-1 is shown above (a) and (b).

        B.   A generalized diagrammatic representation of
   5   the resistant test vectors comprising a
             functional indicator gene having the following
             elements in a 5' to 3' orientation: 1) an HIV-LTR
             U3 region (pLG-luc-HS-1 and pLG-luc-PB-1) or a
             first CMV IE enhancer-promoter (pCG-luc-HS-1 and
  10   pCG-luc-PB-1), 2) the HIV-LTR R and U5 regions,
             3) the coding region of the HIV gag-pol, vif,
             vpr, tat, rev, vpu, deleted env, and nef genes,
             4) a functional indicator gene cassette inserted
             into the deleted env gene, with a transcriptional
  15   orientation opposite to the viral LTRs and 5) a
             3' HIV-LTR.

        C.   A generalized diagrammatic representation of
             the resistance test vectors comprising a
  20   functional indicator gene cassette (pLG-luc-HS-2,
             pLG-luc-PB-2, pCG-luc-HS-2 and pCG-luc-PB-2) in
             which the transcriptional orientation of the
             indicator gene cassette is the same as the viral
             LTRs.

  25

Fig. 7. A.   Demonstration of drug susceptibility using the
              resistance test vectors, pCG-CXCN(F-lucP)2-AA and
              pCG-CXAT(F-lucP)2-AA.   Data are presented as
              luciferase gene activity in target host cells as
  30   Relative Light Units (RLU) in the absence of AZT
              or in the presence of 5 mM AZT.

B.   Drug susceptibility and resistance test performed with the resistance test vector, pCG-CXCN(F-lucP)2-AA containing pre-AZT treatment and post-AZT treatment "test" patient-derived segments. The resistance test vectors are derived from the genomic indicator gene viral vector, pCG-CXCN(F-lucP)2-AA.   Data are presented as percent inhibition of luciferase gene activity in target host cells vs. AZT concentration (log_{10}). pCG-CXCN(F-lucP)2-AA is plotted as solid boxes. pCG-CXCN(F-lucP)2-AA containing patient-derived segments prior to AZT treatment is plotted as solid circles and post-AZT treatment as solid triangles.

C.   Drug susceptibility and resistance test performed with the resistance test vector, pCG-CXCN(F-lucP)2-AA containing the reverse transcriptase segment derived from the biologically active proviral clone, pNL4-3. Data are presented as percent inhibition of luciferase gene activity in target host cells vs. nevirapine concentration (log_{10}) and is plotted as solid boxes.

D.   Drug susceptibility and resistance test performed with the resistance test vector, pCG-CXCN(F-lucP)2-AA containing the protease segment derived from the biologically active proviral clone, pNL4-3. Data are presented as percent inhibition of luciferase gene activity in target host cells vs. indinavir concentration (log_{10}) and is plotted as solid boxes.

Fig. 8 A. Diagrammatic representation of the RNA
pregenomic structure of HBV. Pregenomic RNA is
shown as a solid line. Direct repeat sequences
(DR) are shown as closed rectangles. The
positions of the encapsidation signal sequence is
shown (( ). The C, P, S, and X genes are shown as
open rectangles. The terminal protein (TP),
spacer, DNA polymerase/reverse transcriptase
(pol/RT), and RNase H regions of the P gene are
indicated. Sites of C, P, S, and X translation
initiation are indicated by shaded triangles.

B. A generalized diagrammatic representation of
the subgenomic indicator gene viral vector,
pCS-HBV(NF-IG)II-(PSAS-), a component of the
resistance test vector system, comprising an
indicator gene cassette and an inverted intron
containing the following elements in a 5' to 3'
orientation: (1) the CMV IE enhancer-promoter
region, (2) the 5' region of the HBV genome and
the DR1 and 5' ((the pre-C ORF translation
initiation codon is eliminated), (3) a
non-functional indicator gene cassette in which
the indicator gene ORF contains an inverted
intron, (4) the region of the HBV genome
containing DR2, DR1*, the 3'ε, and the 3' HBV
polyadenylation (pA) signal region.

C. Diagrammatic representation of the covalently
closed circular DNA (cccDNA) form of the
subgenomic indicator gene viral vector,
pCS-HBV(F-IG)II(PSAS-), containing a functional
indicator gene cassette assembled as a result of
HBV viral replication.

-38-

D.   Diagrammatic representation of one example of
     a packaging vector, pPK-CPX, a component of the
     resistance test vector system comprising a
     patient-derived segment containing the following
     elements in a 5' to 3' orientation: (1) the CMV
     IE enhancer-promoter region, (2) the region of
     the HBV genome spanning from the C ORF
     translation initiation codon to the 3' pA signal
     and including the C, P, S, and X genes. The C
     gene of the packaging vector, pPK-CPX, is
     modified such that it does not contain the pre-C
     ORF sequences and does not express the S proteins
     (as shown by the X at the translation initiation
     sites).

E.   Diagrammatic representation of an additional
     packaging vector, pPK-S, providing the S gene
     proteins, that is cotransfected with the
     resistance test vector system comprising the
     indicator   gene   viral   vector,
     pCS-HBV(NF-IG)II-(PSAS-), and the packaging
     plasmid, pPK-CPX.

F.   A generalized diagrammatic representation of
     the   resistance   test   vector,
     pCS-HBV(NF-IG)II-(PSAS+),   comprising   a
     non-functional indicator gene with an inverted
     intron containing the following elements in a 5'
     to   3'   orientation: (1)   the   CMV   IE
     enhancer-promoter region, (2) the 5' region of
     the HBV genome and the DR1 and 5' ((the pre-C
     ORF translation initiation codon is eliminated),
     (3) indicator gene cassette (containing an
     inverted intron) within the region of the HBV
     genome which contains a patient-derived P gene

segment, (4) the region of the HBV genome
containing DR2, DR1*, the 3'ε, and the 3' HBV pA
signal region.

G.   Diagrammatic representation of the covalently
closed circular DNA (cccDNA) form of the
resistance test vector, pCS-HBV(F-IG)II-(PSAS+),
containing a functional indicator gene cassette
and a patient-derived P gene segment assembled as
a result of HBV viral replication.

H.   Diagrammatic representation of a packaging
vector, pPK-CSX, providing the C, S and X gene
proteins, that is cotransfected with the
resistance test vector, pCS-HBV(NF-IG)II-(PSAS+).

Fig. 9 A.   Diagrammatic representation of the RNA
pregenomic structure of HBV.

B.   Diagrammatic representation of an HBV
indicator gene viral vector containing a
non-functional indicator gene cassette. Primer
binding sites for the amplification of a target
DNA sequence are shown. The location and
orientation of the forward primer (Pf) and
reverse primer (Pr) binding sites do not
constitute a functional amplification unit in the
linear form of the vector that is used to
transfect packaging host cells. The Pr binding
site is designed to span the junction sequence
that is generated by splicing of the pregenomic
RNA.

C.   Diagrammatic representation of the rc-DNA form of the indicator gene viral vector described in 10B. Primer binding sites for the amplification of a target DNA sequence are shown. The location and orientation of the Pf and Pr primer binding sites constitute a functional amplification unit in the plus strand DNA component of the rc-DNA form and the plus and minus strand DNA components of the cccDNA form of the vector that is generated by HBV DNA replication within the virus particles produced in packaging host cells. The Pr binding site is assembled by the splicing of the pregenomic RNA.

D.   Diagrammatic representation of an HBV indicator gene viral vector containing a non-functional indicator gene cassette. Primer binding sites for the amplification of a target DNA sequence are shown. The location and orientation of the Pf and Pr binding sites constitute a functional amplification unit in the linear form of the vector that is used to transfect packaging host cells, but the Pf binding site is not adjacent to the binding site of the exonuclease detection probe (probe) in the unspliced linear form of the vector. This arrangement of Pf, Pr, and probe binding sites does not constitute an efficient exonuclease detection unit.

-41-

E.   Diagrammatic representation of the rc-DNA form
      of the indicator gene viral vector described in
      Fig. 9D.   Primer binding sites for the
      amplification of a target DNA sequence are shown.
      The location and orientation of the Pf and Pr
      primer binding sites constitute a functional
      amplification unit in the rc-DNA and cccDNA forms
      of the vector that are generated by HBV DNA
      replication in packaging host cells. The location
      of the Pf binding site is brought immediately
      adjacent to the binding site of the exonuclease
      detection probe (probe) in the rc-DNA and cccDNA
      forms of the vector. This arrangement of Pf, Pr,
      and probe binding sites constitute an efficient
      exonuclease detection unit.

F.   Diagrammatic representation of an HBV
      indicator gene viral vector. Primer binding
      sites for the amplification of a target DNA
      sequence are shown. The location and orientation
      of the forward primer (Pf) and reverse primer
      (Pr) binding sites do not constitute a functional
      amplification unit in the linear form of the
      vector that is used to transfect packaging host
      cells.

G.   Diagrammatic representation of the rc-DNA form
      of the indicator gene viral vector described in
      10F. Primer binding sites for the amplification
      of a target DNA sequence are shown. The location
      and orientation of the Pf and Pr primer binding
      sites constitute a functional amplification unit
      in the plus strand DNA component of the rc-DNA
      form and the plus and minus strand DNA components

-42-

of the cccDNA form of the vector that is generated by HBV DNA replication within the virus particles produced in packaging host cells.

5   Fig. 10 A.   Diagrammatic representation of the RNA pregenomic structure of HBV.

B.   A generalized diagrammatic representation of the subgenomic indicator gene viral vector, pCS-HBV(NF-IG)PP-(PSAS-), a component of the resistance test vector system comprising a non-functional indicator gene with a permuted promoter containing the following elements in a 5' to 3' orientation: (1) the CMV IE enhancer promoter region, (2) the 5' region of the HBV genome and the DR1 and 5' ((the pre-C ORF translation initiation codon is eliminated), (3) a non-functional indicator gene cassette assembled such that the promoter region is positioned 3', i.e. downstream, of the indicator gene ORF, (4) the 3' region of the HBV genome containing DR2, DR1*, the 3'ε, and the 3' HBV pA signal region (the pre-C ORF translation initiation codon is eliminated). The packaging vector, pPK-CPX, a component of the resistance test vector system comprising a patient-derived P gene segment is shown in Fig. 8D and the S packaging vector, pPK-S, is shown in Fig. 8E.

C.   Diagrammatic representation of the covalently closed circular DNA (cccDNA) form of the subgenomic indicator gene viral vector, pCS-HBV(F-IG)PP(PSAS-), containing a functional indicator gene cassette assembled as a result of HBV viral replication.

-43-

D.   A generalized diagrammatic representation of
the   resistance   test   vector,
pCS-HBV(NF-IG)PP(PSAS+),   comprising   a
non-functional indicator gene with a permuted
promoter containing the following elements in a
5' to 3' orientation: (1) the CMV IE
enhancer-promoter region, (2) the 5' region of
the HBV genome and the DR1 and 5' copy of ((the
pre-C ORF translation initiation codon is
eliminated) (3) an enhancer-promoter region
(permuted promoter), (4) the P gene containing
the patient-derived segment (5) the indicator
gene ORF (6) an internal ribosome entry site
(IRES), and (7) the 3' region of the HBV genome
containing DR2, DR1*, the 3'ε, and the 3' HBV pA
signal region (the pre-C ORF translation
initiation codon is eliminated). The packaging
vector, pPK-CSX, providing the C, S and X genes
is cotransfected with the resistance test vector,
pCS-HBV(NF-IG)PP(PSAS+), and is shown in Fig. 8H.

E.   Diagrammatic representation of the covalently
closed circular DNA (cccDNA) form of the
resistance test vector, pCS-HBV(F-IG)PP(PSAS+),
containing a functional indicator gene cassette
and a patient-derived P gene segment assembled as
a result of HBV viral replication.

Fig. 11 A.   Diagrammatic representation of the RNA
pregenomic structure of HBV.

B.   A generalized diagrammatic representation of the subgenomic indicator gene viral vector, pCS-HBV(NF-IG)PPTIS-(PSAS-), a component of the resistance test vector system comprising a non-functional indicator gene with a permuted promoter and translation initiation site containing the following elements in a 5' to 3' orientation: (1) the CMV IE enhancer-promoter region, (2) the 5' region of the HBV genome including the DR1 and 5' ((the pre-C ORF translation initiation codon is eliminated), (3) an indicator gene ORF lacking a translation initiation site, (4) an enhancer-promoter region (permuted promoter) (5) the 3' region of the HBV genome containing DR2, pre-C ORF translation initiation codon, DR1*, the 3'ε, and the 3' HBV pA signal region. The packaging vector, pPK-CPX, a component of the resistance test vector system comprising a patient-derived segment is shown in Fig. 8D and the S packaging vector, pPK-S, is shown in Fig. 8E.

C.   Diagrammatic representation of the covalently closed circular DNA (cccDNA) form of the subgenomic indicator gene viral vector, pCS-HBV(F-IG)PPTIS(PSAS-), containing a functional indicator gene cassette assembled as a result of HBV viral replication.

D.   A generalized diagrammatic representation of the resistance test vector, pCS-HBV(NF-IG)PPTIS(PSAS+), comprising a non-functional indicator gene with a permuted promoter containing the following elements in a 5' to 3' orientation: (1) the CMV IE

-45-

enhancer-promoter region, (2) the 5' region of
the HBV genome including the DR1 and 5' ((the
pre-C ORF translation initiation codon is
eliminated), (3) an indicator gene ORF lacking a
translation initiation site, (4) the P gene
containing the patient-derived segment, (5) an
enhancer-promoter region (permuted promoter) (6)
the 3' region of the HBV genome containing DR2,
pre-C ORF translation initiation codon, DR1*, the
3'ε, and the 3' HBV pA signal region.   The
packaging vector, pPK-CSX, providing the C, S and
X genes is cotransfected with the resistance test
vector, pCS-HBV(NF-IG)PPTIS(PSAS+), and is shown
in Fig. 8H.

E.   Diagrammatic representation of the covalently
closed circular DNA (cccDNA) form of the
r e s i s t a n c e   t e s t   v e c t o r ,
pCS-HBV(F-IG)PPTIS(PSAS+),   containing   a
functional   indicator   gene   cassette   and   a
patient-derived P gene segment assembled as a
result of HBV viral replication.

Fig. 12 A.   Diagrammatic   representation   of   the   RNA
pregenomic structure of HBV.

B.   A generalized diagrammatic representation of
the subgenomic indicator gene viral vector,
pCS-HBV(NF-IG)PCR-(PSAS-), a component of the
resistance test vector system comprising a
non-functional indicator gene with a permuted
coding region containing the following elements
in a 5' to 3' orientation: (1) the CMV IE
enhancer-promoter region, (2) the 5' region of
the HBV genome including the DR1 and 5' ((the

pre-C ORF translation initiation codon is eliminated), (3) a non-functional indicator gene cassette assembled such that the promoter region and a 5' portion of the coding region are positioned 3', i.e. downstream, of the remaining 3' portion of the coding region, (4) the 3' region of the HBV genome containing DR2, DR1*, the 3'ε, and the 3' HBV pA signal region (the pre-C ORF translation initiation codon is eliminated). The packaging vector, pPK-CPX, a component of the resistance test vector system, comprising a patient-derived P gene segment is shown in Fig. 8D and the S packaging vector, pPK-S, is shown in Fig. 8E.

C. Diagrammatic representation of the covalently closed circular DNA (cccDNA) form of the subgenomic indicator gene viral vector, pCS-HBV(F-IG)PCR(PSAS-), containing a functional indicator gene cassette assembled as a result of HBV viral replication.

D. A generalized diagrammatic representation of the resistance test vector, pCS-HBV(NF-IG)PCR(PSAS+), comprising a non-functional indicator gene with a permuted coding region containing the following elements in a 5' to 3' orientation: (1) the CMV IE enhancer-promoter region, (2) the 5' region of the HBV genome including the DR1 and 5' ((the pre-C ORF translation initiation codon is eliminated), (3) the 3' portion of the indicator gene ORF beginning with a splice acceptor sequence in the reverse orientation, (4) the P gene containing the patient-derived segment, (5)

an enhancer-promoter region, (6) the 5' portion
of the indicator gene ORF ending in a splice
donor sequence in the reverse orientation, (7)
the 3' region of the HBV genome containing DR2,
5   DR1*, the 3'ε, and the 3' HBV pA signal region
(the pre-C ORF translation initiation codon is
eliminated). The packaging vector, pPK-CSX,
providing the C, S and X genes is cotransfected
with   the   resistance   test   vector,
10   pCS-HBV(NF-IG)PCR(PSAS+), and is shown in Fig.
8H.

E.   Diagrammatic representation of the covalently
closed circular DNA (cccDNA) form of the
15   resistance test vector, pCS-HBV(F-IG)PCR(PSAS+),
containing a functional indicator gene cassette
and a patient-derived P gene segment assembled as
a result of HBV viral replication.

20   Fig. 13 A.   Diagrammatic representation of the RNA
pregenomic structure of HBV.

B.   A generalized diagrammatic representation of
the subgenomic indicator gene viral vector
25   component, pCS-HBV(F-IG)(PSAS-), a component of
the resistance test vector system comprising a
functional indicator gene cassette containing the
following elements in a 5' to 3' orientation: (1)
the CMV IE enhancer-promoter region, (2) the 5'
30   region of the HBV genome including the DR1 and 5'
((the pre-C ORF translation initiation codon is
eliminated), (3) a functional indicator gene
cassette, (4) the 3' region of the HBV genome
containing DR2, DR1*, the 3'ε, and the 3' HBV pA
35   signal region (the pre-C ORF translation

initiation codon is eliminated). The packaging vector, pPK-CPX, a component of the resistance test vector system comprising a patient-derived P gene segment is shown in Fig. 8D and the S packaging vector, pPK-S, is shown in Fig. 8E.

C. Diagrammatic representation of the covalently closed circular DNA (cccDNA) form of the subgenomic indicator gene viral vector, pCS-HBV(F-IG)(PSAS-), containing a functional indicator gene.

D. A generalized diagrammatic representation of the resistance test vector, pCS-HBV(F-IG)(PSAS+), comprising a functional indicator gene containing the following elements in a 5' to 3' orientation: (1) the CMV IE enhancer promoter region, (2) the 5' region of the HBV genome including the DR1 and 5' ε (the pre-C ORF translation initiation codon is eliminated), (3) a functional indicator gene cassette, (4) the P gene containing the patient-derived segment, (5) the 3' region of the HBV genome containing DR2, DR1*, the 3'ε, and the 3' HBV pA signal region (the pre-C ORF translation initiation codon is eliminated). The packaging vector, pPK-CSX, providing the C, S and X genes, that is cotransfected with the resistance test vector, pCS-HBV(F-IG)(PSAS+), and is shown in Fig. 8H.

E. Diagrammatic representation of the covalently closed circular DNA (cccDNA) form of the resistance test vector, pCS-HBV(F-IG)(PSAS+), containing a functional indicator gene.

Detailed Description of the Invention

In order that the invention described herein may be more
fully understood, the following description is set forth.

5   The present invention provides a novel drug susceptibility
       and resistance assay comprising the steps of: (a)
       introducing a resistance test vector comprising a
       patient-derived segment and an indicator gene into a host
       cell; (b) culturing the host cell from step (a); (c)
10   measuring expression of the indicator gene in a target host
       cell; and (d) comparing the expression of the indicator gene
       from step (c) with the expression of the indicator gene
       measured when steps (a)-(c) are carried out in the absence
       of the anti-viral drug, wherein a test concentration of the
15   anti-viral drug is present at steps (a)-(c); at steps
       (b)-(c); or at step (c).

       In one aspect of the invention there is provided a method
       for determining susceptibility for an anti-viral drug
20   comprising: (a) introducing a resistance test vector
       comprising a patient-derived segment and an indicator gene
       into a host cell; (b) culturing the host cell from step (a);
       (c) measuring an indicator in a target host cell wherein
       said indicator is a DNA or RNA structure; and (d) comparing
25   the measurement of the indicator from step (c) with the
       measurement of the indicator when steps (a)-(c) are carried
       out in the absence of the anti-viral drug, wherein a test
       concentration of the anti-viral drug is present at steps
       (a)-(c); at steps (b)-(c); or at step (c).
30
       This invention also provides a method for determining
       anti-viral drug resistance in a patient comprising:(a)
       developing a standard curve of drug susceptibility for an
       anti-viral drug; (b) determining anti-viral drug
35   susceptibility in the patient using either of the

susceptibility tests described above; and (c) comparing the anti-viral drug susceptibility in step (b) with the standard curve determined in step (a), wherein a decrease in anti-viral susceptibility indicates development of anti-viral drug resistance in the patient.

This invention also provides a method for determining anti-viral drug resistance in a patient comprising: (a) determining anti-viral drug susceptibility in the patient at a first time according to either of the above methods, wherein the patient-derived segment is obtained from the patient at about said time; (b) determining anti-viral drug susceptibility of the same patient at a later time; and (c) comparing the anti-viral drug susceptibilities determined in step (a) and (b), wherein a decrease in anti-viral drug susceptibility at the later time compared to the first time indicates development or progression of anti-viral drug resistance in the patient.

The assay of this invention can be used for any viral disease where anti-viral drug susceptibility and resistance is a concern including, for example, HIV, herpes simplex virus, cytomegalovirus virus, varicella zoster virus, other human herpes (HIV) viruses, influenza A virus, respiratory syncytial virus, hepatitis A, B and C viruses, rhinovirus, and human papilloma virus. The foregoing are representative of certain viruses for which there is presently available anti-viral chemotherapy, and represent the viral families retroviridae, herpesviridae, orthomyxoviridae, pneumovirus and hepadnaviridae. The assay of this invention would be used with other viral infections arising from infections due to other viruses within these families as well as viral infections arising from viruses in other viral families. In addition, the drug susceptibility and resistance test of this invention is useful for screening for compounds to

-51-

treat viral diseases for which there is no currently
available therapy.

The structure, life cycle and genetic elements of the
5   viruses which could be tested in the drug susceptibility and
resistance test of this invention would be known to one of
ordinary skill in the art. It is useful to the practice of
this invention, for example, to understand the life cycle of
a retrovirus, as well as the viral genes required for
10   retrovirus rescue and infectivity. Retrovirally infected
cells shed a membrane virus containing a diploid RNA genome.
The virus, studded with an envelope glycoprotein (which
serves to determine the host range of infectivity), attaches
to a cellular receptor in the plasma membrane of the cell to
15   be infected. After receptor binding, the virus is
internalized and uncoated as it passes through the cytoplasm
of the host cell. Either on its way to the nucleus or in
the nucleus, the reverse transcriptase molecules resident in
the viral core drive the synthesis of the double-stranded
20   DNA provirus, a synthesis that is primed by the binding of
a tRNA molecule to the genomic viral RNA. The
double-stranded DNA provirus is subsequently integrated in
the genome of the host cell, where it can serve as a
transcriptional template for both mRNAs encoding viral
25   proteins and virion genomic RNA, which will be packaged into
viral core particles. On their way out of the infected
cell, core particles move through the cytoplasm, attach to
the inside of the plasma membrane of the newly infected
cell, and bud, taking with them tracts of membrane
30   containing the virally encoded envelope glycoprotein gene
product. This cycle of infection - reverse transcription,
transcription, translation, virion assembly, and budding -
repeats itself over and over again as infection spreads.

35   The viral RNA and, as a result, the proviral DNA encode

-52-

several cis-acting elements that are vital to the successful completion of the viral lifecycle. The virion RNA carries the viral promoter at its 3' end. Replicative acrobatics place the viral promoter at the 5' end of the proviral genome as the genome is reverse transcribed. Just 3' to the 5' retroviral LTR lies the viral packaging site. The retroviral lifecycle requires the presence of virally encoded transacting factors. The viral-RNA-dependent DNA polymerase (pol)-reverse transcriptase is also contained within the viral core and is vital to the viral life cycle in that it is responsible for the conversion of the genomic RNA to the integrative intermediate proviral DNA. The viral envelope glycoprotein, env, is required for viral attachment to the uninfected cell and for viral spread. There are also transcriptional trans-activating factors, so called transactivators, that can serve to modulate the level of transcription of the integrated parental provirus. Typically, replication-competent (non-defective) viruses are self-contained in that they encode all of these trans-acting factors. Their defective counterparts are not self-contained.

In the case of a DNA virus, such as a hepadnavirus, understanding the life cycle and viral genes required for infection is useful to the practice of this invention. The process of HBV entry has not been well defined. Replication of HBV uses an RNA intermediate template. In the infected cell the first step in replication is the conversion of the asymmetric relaxed circle DNA (rc-DNA) to covalently closed circle DNA (cccDNA). This process, which occurs within the nucleus of infected liver cells, involves completion of the DNA positive-strand synthesis and ligation of the DNA ends. In the second step, the cccDNA is transcribed by the host RNA polymerase to generate a 3.5 kB RNA template (the pregenome). This pregenome is complexed with protein in the

viral core. The third step involves the synthesis of the
first negative-sense DNA strand by copying the pregenomic
RNA using the virally encoded P protein reverse
transcriptase. The P protein also serves as the minus
5   strand DNA primer. Finally, the synthesis of the second
positive-sense DNA strand occurs by copying the first DNA
strand, using the P protein DNA polymerase activity and an
oligomer of viral RNA as primer. The pregenome also
transcribes mRNA for the major structural core proteins.

10

The following flow chart illustrates certain of the various
vectors and host cells which may be used in this invention.
It is not intended to be all inclusive.

Vectors

15   Indicator gene cassette   +   Viral vector
     (functional/nonfunctional   (genomic or subgenomic)
      indicator gene)

                                    ↓

20

                    Indicator Gene Viral Vector
             (functional/nonfunctional indicator gene)

25 | + Patient sequence
                                        | acceptor sites
                                        |
                                        | + Patient-derived
                                        | segments
30   ↓

                    Resistance Test Vector
          (patient-derived segments + indicator gene)

35   Host Cells

     Packaging Host Cell - transfected with packaging
     expression vectors

40   Resistance Test Vector Host Cell - a packaging host cell
     transfected with a resistance test vector

     Target Host Cell - a host cell to be infected by a
     resistance test vector viral particle produced by the
45   resistance test vector host cell

Resistance Test Vector

"Resistance test vector" means one or more vectors which taken together contain DNA or RNA comprising a patient-derived segment and an indicator gene. In the case where the resistance test vector comprises more than one vector the patient-derived segment may be contained in one vector and the indicator gene in a different vector. Such a resistance test vector comprising more than one vector is referred to herein as a resistance test vector system for purposes of clarity but is nevertheless understood to be a resistance test vector. The DNA or RNA of a resistance test vector may thus be contained in one or more DNA or RNA molecules. In one embodiment, the resistance test vector is made by insertion of a patient-derived segment into an indicator gene viral vector. In another embodiment, the resistance test vector is made by insertion of a patient-derived segment into a packaging vector while the indicator gene is contained in a second vector, for example an indicator gene viral vector. As used herein, "patient-derived segment" refers to one or more viral segments obtained directly from a patient using various means, for example, molecular cloning or polymerase chain reaction (PCR) amplification of a population of patient-derived segments using viral DNA or complementary DNA (cDNA) prepared from viral RNA, present in the cells (e.g. peripheral blood mononuclear cells, PBMC), serum or other bodily fluids of infected patients. When a viral segment is "obtained directly" from a patient it is obtained without passage of the virus through culture, or if the virus is cultured, then by a minimum number of passages to essentially eliminate the selection of mutations in culture. The term "viral segment" refers to any functional viral sequence or viral gene encoding a gene product (e.g., a

protein) that is the target of an anti-viral drug. The term "functional viral sequence" as used herein refers to any nucleic acid sequence (DNA or RNA) with functional activity such as enhancers, promoters, polyadenylation sites, sites of action of trans-acting factors, such as tar and RRE, packaging sequences, integration sequences, or splicing sequences. If a drug were to target more than one functional viral sequence or viral gene product then patient-derived segments corresponding to each said viral gene would be inserted in the resistance test vector. In the case of combination therapy where two or more anti-virals targeting two different functional viral sequences or viral gene products are being evaluated, patient-derived segments corresponding to each functional viral sequence or viral gene product would be inserted in the resistance test vector. The patient-derived segments are inserted into unique restriction sites or specified locations, called patient sequence acceptor sites, in the indicator gene viral vector or for example, a packaging vector depending on the particular construction being used as described herein.

As used herein, "patient-derived segment" encompasses segments derived from human and various animal species. Such species include, but are not limited to chimpanzees, horses, cattles, cats and dogs.

Patient-derived segments can also be incorporated into resistance test vectors using any of several alternative cloning techniques. For example, cloning via the introduction of class II restriction sites into both the plasmid backbone and the patient-derived segments or by uracil DNA glycosylase primer cloning (refs).

The patient-derived segment may be obtained by any method of

-56-

molecular cloning or gene amplification, or modifications
thereof, by introducing patient sequence acceptor sites, as
described below, at the ends of the patient-derived segment
to be introduced into the resistance test vector.   For
5   example, in a gene amplification method such as PCR,
restriction sites corresponding to the patient-sequence
acceptor sites can be incorporated at the ends of the
primers used in the PCR reaction. Similarly, in a molecular
cloning method such as cDNA cloning, said restriction sites
10 can be incorporated at the ends of the primers used for
first or second strand cDNA synthesis, or in a method such
as primer-repair of DNA, whether cloned or uncloned DNA,
said restriction sites can be incorporated into the primers
used for the repair reaction. The patient sequence acceptor
15 sites and primers are designed to improve the representation
of patient-derived segments.   Sets of resistance test
vectors having designed patient sequence acceptor sites
provide representation of patient-derived segments that
would be underrepresented in one resistance test vector
20 alone.

Resistance test vectors are prepared by modifying an
indicator gene viral vector (described below) by introducing
patient sequence acceptor sites, amplifying or cloning
25 patient-derived segments and inserting the amplified or
cloned sequences precisely into indicator gene viral vectors
at the patient sequence acceptor sites.   The resistance
test vectors are constructed from indicator gene viral
vectors which are in turn derived from genomic viral vectors
30 or subgenomic viral vectors and an indicator gene cassette,
each of which is described below. Resistance test vectors
are then introduced into a host cell.   Alternatively, a
resistance test vector (also referred to as a resistance
test vector system) is prepared by introducing patient
35 sequence acceptor sites into a packaging vector, amplifying

-57-

or cloning patient-derived segments and inserting the
amplified or cloned sequences precisely into the packaging
vector at the patient sequence acceptor sites and
co-transfecting this packaging vector with an indicator gene
5   viral vector.

In one preferred embodiment, the resistance test vector may
be introduced into packaging host cells together with
packaging expression vectors, as defined below, to produce
10   resistance test vector viral particles that are used in drug
resistance and susceptibility tests that are referred to
herein as a "particle-based test." In an alternative
preferred embodiment, the resistance test vector may be
introduced into a host cell in the absence of packaging
15   expression vectors to carry out a drug resistance and
susceptibility test that is referred to herein as a
"non-particle-based test." As used herein a "packaging
expression vector" provides the factors, such as packaging
proteins (e.g. structural proteins such as core and envelope
20   polypeptides), transacting factors, or genes required by
replication-defective retrovirus or hepadnavirus. In such
a situation, a replication-competent viral genome is
enfeebled in a manner such that it cannot replicate on its
own. This means that, although the packaging expression
25   vector can produce the trans-acting or missing genes
required to rescue a defective viral genome present in a
cell containing the enfeebled genome, the enfeebled genome
cannot rescue itself.

30   *Indicator or Indicator Gene*
"Indicator or indicator gene" refers to a nucleic acid
encoding a protein, DNA or RNA structure that either
directly or through a reaction gives rise to a measurable or
noticeable aspect, e.g. a color or light of a measurable
35   wavelength or in the case of DNA or RNA used as an indicator

a change or generation of a specific DNA or RNA structure. Preferred examples of an indicator gene is the *E. coli lacZ* gene which encodes beta-galactosidase, the *luc* gene which encodes luciferase either from, for example, *Photonis pyralis* (the firefly) or *Renilla reniformis* (the sea pansy), the *E. coli phoA* gene which encodes alkaline phosphatase, green fluorescent protein and the bacterial CAT gene which encodes chloramphenicol acetyltransferase. Additional preferred examples of an indicator gene are secreted proteins or cell surface proteins that are readily measured by assay, such as radioimmunoassay (RIA), or fluorescent activated cell sorting (FACS), including, for example, growth factors, cytokines and cell surface antigens (e.g. growth hormone, Il-2 or CD4, respectively). "Indicator gene" is understood to also include a selection gene, also referred to as a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase, hygromycin, neomycin, zeocin or *E. coli* gpt. In the case of the foregoing examples of indicator genes, the indicator gene and the patient-derived segment are discrete, i.e. distinct and separate genes. In some cases a patient-derived segment may also be used as an indicator gene. In one such embodiment in which the patient-derived segment corresponds to more than one viral gene which is the target of an anti-viral, one of said viral genes may also serve as the indicator gene. For example, a viral protease gene may serve as an indicator gene by virtue of its ability to cleave a chromogenic substrate or its ability to activate an inactive zymogen which in turn cleaves a chromogenic substrate, giving rise in each case to a color reaction. In all of the above examples of indicator genes, the indicator gene may be either "functional" or "non-functional" but in each case the expression of the indicator gene in the target cell is ultimately dependent upon the action of the patient-derived

-59-

segment.

Functional Indicator Gene

In the case of a "functional indicator gene" the indicator
gene may be capable of being expressed in a "packaging host
cell/resistance test vector host cell" as defined below,
independent of the patient-derived segment, however the
functional indicator gene could not be expressed in the
target host cell, as defined below, without the production
of functional resistance test vector particles and their
effective infection of the target host cell. In one
embodiment of a functional indicator gene, the indicator
gene cassette, comprising control elements and a gene
encoding an indicator protein, is inserted into the
indicator gene viral vector with the same or opposite
transcriptional orientation as the native or foreign
enhancer/promoter of the viral vector. One example of a
functional indicator gene in the case of HIV or HBV, places
the indicator gene and its promoter (a CMV IE
enhancer/promoter) in the same or opposite transcriptional
orientation as the HIV-LTR or HBV enhancer-promoter,
respectively, or the CMV IE enhancer/promoter associated
with the viral vector.

Non-Functional Indicator Gene

Alternatively the indicator gene, may be "non-functional" in
that the indicator gene is not efficiently expressed in a
packaging host cell transfected with the resistance test
vector, which is then referred to a resistance test vector
host cell, until it is converted into a functional indicator
gene through the action of one or more of the
patient-derived segment products. An indicator gene is
rendered non-functional through genetic manipulation
according to this invention.

1. <u>Permuted Promoter</u> In one embodiment an indicator gene is rendered non-functional due to the location of the promoter, in that, although the promoter is in the same transcriptional orientation as the indicator gene, it follows rather than precedes the indicator gene coding sequence. This misplaced promoter is referred to as a "permuted promoter." In addition to the permuted promoter the orientation of the non-functional indicator gene is opposite to that of the native or foreign promoter/enhancer of the viral vector. Thus the coding sequence of the non-functional indicator gene can neither be transcribed by the permuted promoter nor by the viral promoters. The non-functional indicator gene and its permuted promoter is rendered functional by the action of one or more of the viral proteins. One example of a non-functional indicator gene with a permuted promoter in the case of HIV, places a T7 phage RNA polymerase promoter (herein referred to as T7 promoter) promoter in the 5' LTR in the same transcriptional orientation as the indicator gene. The indicator gene cannot be transcribed by the T7 promoter as the indicator gene cassette is positioned upstream of the T7 promoter. The non-functional indicator gene in the resistance test vector is converted into a functional indicator gene by reverse transcriptase upon infection of the target cells, resulting from the repositioning of the T7 promoter, by copying from the 5' LTR to the 3' LTR, relative to the indicator gene coding region. Following the integration of the repaired indicator gene into the target cell chromosome by HIV integrase, a nuclear T7 RNA polymerase expressed by the target cell transcribes the indicator gene. One example of a non-functional indicator gene with a permuted promoter in the case of HBV, places an enhancer-promoter region downstream or 3' of the indicator gene both having the same transcriptional orientation. The indicator gene cannot be transcribed by the enhancer-promoter as the indicator gene

cassette is positioned upstream. The non-functional indicator gene in the resistance test vector is converted into a functional indicator gene by reverse transcription and circularization of the HBV indicator gene viral vector by the repositioning of the enhancer-promoter upstream relative to the indicator gene coding region.

A permuted promoter may be any eukaryotic or prokaryotic promoter which can be transcribed in the target host cell. Preferably the promoter will be small in size to enable insertion in the viral genome without disturbing viral replication. More preferably, a promoter that is small in size and is capable of transcription by a single subunit RNA polymerase introduced into the target host cell, such as a bacteriophage promoter, will be used. Examples of such bacteriophage promoters and their cognate RNA polymerases include those of phages T7, T3 and Sp6. A nuclear localization sequence (NLS) may be attached to the RNA polymerase to localize expression of the RNA polymerase to the nucleus where they may be needed to transcribed the repaired indicator gene. Such an NLS may be obtained from any nuclear-transported protein such as the SV40 T antigen. If a phage RNA polymerase is employed, an internal ribosome entry site (IRES) such as the EMC virus 5' untranslated region (UTR) may be added in front of the indicator gene, for translation of the transcripts which are generally uncapped. In the case of HIV, the permuted promoter itself can be introduced at any position within the 5' LTR that is copied to the 3' LTR during reverse transcription so long as LTR function is not disrupted, preferably within the U5 and R portions of the LTR, and most preferably outside of functionally important and highly conserved regions of U5 and R. In the case of HBV, the permuted promoter can be placed at any position that does not disrupt the cis acting elements that are necessary for HBV DNA replication.

Blocking sequences may be added at the ends of the resistance test vector should there be inappropriate expression of the non-functional indicator gene due to transfection artifacts (DNA concatenation). In the HIV example of the permuted T7 promoter given above, such a blocking sequence may consist of a T7 transcriptional terminator, positioned to block readthrough transcription resulting from DNA concatenation, but not transcription resulting from repositioning of the permuted T7 promoter from the 5' LTR to the 3' LTR during reverse transcription.

2. Permuted Coding Region   In a second embodiment, an indicator gene is rendered non-functional due to the relative location of the 5' and 3' coding regions of the indicator gene, in that, the 3' coding region precedes rather than follows the 5' coding region. This misplaced coding region is referred to as a "permuted coding region." The orientation of the non-functional indicator gene may be the same or opposite to that of the native or foreign promoter/enhancer of the viral vector, as mRNA coding for a functional indicator gene will be produced in the event of either orientation. The non-functional indicator gene and its permuted coding region is rendered functional by the action of one or more of the patient-derived segment products. A second example of a non-functional indicator gene with a permuted coding region in the case of HIV, places a 5' indicator gene coding region with an associated promoter in the 3' LTR U3 region and a 3' indicator gene coding region in an upstream location of the HIV genome, with each coding region having the same transcriptional orientation as the viral LTRs. In both examples, the 5' and 3' coding regions may also have associated splice donor and acceptor sequences, respectively, which may be heterologous or artificial splicing signals. The indicator gene cannot be functionally transcribed either by the associated

-63-

promoter or viral promoters, as the permuted coding region
prevents the formation of functionally spliced transcripts.
The non-functional indicator gene in the resistance test
vector is converted into a functional indicator gene by
reverse transcriptase upon infection of the target cells,
resulting from the repositioning of the 5' and 3' indicator
gene coding regions relative to one another, by copying of
the 3' LTR to the 5' LTR. Following transcription by the
promoter associated with the 5' coding region, RNA splicing
can join the 5' and 3' coding regions to produce a
functional indicator gene product. One example of a
non-functional indicator gene with a permuted coding region
in the case of HBV, places a 3' indicator gene coding region
upstream or 5' of the enhancer-promoter and the 5' coding
region of the indicator gene. The transcriptional
orientation of the indicator gene 5' and 3' coding regions
are identical to one another, and the same as that of the
indicator gene viral vector. However, as the indicator gene
5' and 3' coding regions are permuted in the resistance test
vectors (i.e., the 5' coding region is downstream of the 3'
coding region), no mRNA is transcribed which can be spliced
to generate a functional indicator gene coding region.
Following reverse transcription and circularization of the
indicator gene viral vector, the indicator gene 3' coding
region is positioned downstream or 3' to the
enhancer-promoter and 5' coding regions thus permitting the
transcription of mRNA which can be spliced to generate a
functional indicator gene coding region.

3. Inverted Intron In a third embodiment, the indicator
gene is rendered non-functional through use of an "inverted
intron," i.e. an intron inserted into the coding sequence of
the indicator gene with a transcriptional orientation
opposite to that of the indicator gene. The overall
transcriptional orientation of the indicator gene cassette

-64-

including its own, linked promoter, is opposite to that of
the viral control elements, while the orientation of the
artificial intron is the same as the viral control elements.
Transcription of the indicator gene by its own linked
promoter does not lead to the production of functional
transcripts as the inverted intron cannot be spliced in this
orientation.   Transcription of the indicator gene by the
viral control elements does, however, lead to the removal of
the inverted intron by RNA splicing, although the indicator
gene is still not functionally expressed as the resulting
transcript has an antisense orientation.   Following the
reverse transcription of this transcript and integration of
the resultant retroviral DNA, or the circularization of
hepadnavirus DNA, the indicator gene can be functionally
transcribed using its own linked promoter as the inverted
intron has been previously removed.   In this case, the
indicator gene itself may contain its own functional
promoter with the entire transcriptional unit oriented
opposite to the viral control elements.   Thus the
non-functional indicator gene is in the wrong orientation to
be transcribed by the viral control elements and it cannot
be functionally transcribed by its own promoter, as the
inverted intron cannot be properly excised by splicing.
However, in the case of a retrovirus and HIV specifically
and hepadnaviruses, and HBV specifically, transcription by
the viral promoters (HIV LTR or HBV enhancer-promoter)
results in the removal of the inverted intron by splicing.
As a consequence of reverse transcription of the resulting
spliced transcript and the integration of the resulting
provirus into the host cell chromosome or circularization of
the HBV vector, the indicator gene can now be functionally
transcribed by its own promoter.   The inverted intron,
consisting of a splice donor and acceptor site to remove the
intron, is preferably located in the coding region of the
indicator gene in order to disrupt translation of the

-65-

indicator gene. The splice donor and acceptor may be any splice donor and acceptor. A preferred splice donor-receptor is the CMV IE splice donor and the splice acceptor of the second exon of the human alpha globin gene ("intron A").

Indicator Gene Viral Vector - Construction

As used herein, "indicator gene viral vector" refers to a vector(s) comprising an indicator gene and its control elements and one or more viral genes. The indicator gene viral vector is assembled from an indicator gene cassette and a "viral vector," defined below. The indicator gene viral vector may additionally include an enhancer, splicing signals, polyadenylation sequences, transcriptional terminators, or other regulatory sequences. Additionally the indicator gene viral vector may be functional or nonfunctional. In the event that the viral segments which are the target of the anti-viral drug are not included in the indicator gene viral vector they are provided in a second vector. An "indicator gene cassette" comprises an indicator gene and control elements. "Viral vector" refers to a vector comprising some or all of the following: viral genes encoding a gene product, control sequences, viral packaging sequences, and in the case of a retrovirus, integration sequences. The viral vector may additionally include one or more viral segments one or more of which may be the target of an anti-viral drug. Two examples of a viral vector which contain viral genes are referred to herein as an "genomic viral vector" and a "subgenomic viral vector." A "genomic viral vector" is a vector which may comprise a deletion of a one or more viral genes to render the virus replication incompetent, but which otherwise preserves the mRNA expression and processing characteristics of the complete virus. In one embodiment for an HIV drug susceptibility and resistance test, the genomic viral vector

comprises the HIV *gag-pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef* genes (some, most or all of *env* may be deleted). A "subgenomic viral vector" refers to a vector comprising the coding region of one or more viral genes which may encode the proteins that are the target(s) of the anti-viral drug. In the case of HIV, a preferred embodiment is a subgenomic viral vector comprising the HIV *gag-pol* gene. In the case of HBV a preferred embodiment is a subgenomic viral vector comprising the HBV P gene. In the case of HIV, two examples of proviral clones used for viral vector construction are HXB2 (Fisher et al., (1986) *Nature*, **320**, 367-371) and NL4-3 (Adachi et al., (1986) *J. Virol.*, **59**, 284-291). In the case of HBV, a large number of full length genomic sequences have been characterized and could be used for construction of HBV viral vectors: GenBank Nos. M54923, M38636, J02203 and X59795. The viral coding genes may be under the control of a native enhancer/promoter or a foreign viral or cellular enhancer/promoter. A preferred embodiment for an HIV drug susceptibility and resistance test, is to place the genomic or subgenomic viral coding regions under the control of the native enhancer/promoter of the HIV-LTR U3 region or the CMV immediate-early (IE) enhancer/promoter. A preferred embodiment for an HBV drug susceptibility and resistance test, is to place the genomic or subgenomic viral coding regions under the control of the CMV immediate-early (IE) enhancer/promoter. In the case of an indicator gene viral vector that contains one or more viral genes which are the targets or encode proteins which are the targets of an anti-viral drug(s) then said vector contains the patient sequence acceptor sites. The patient-derived segments are inserted in the patient sequence acceptor site in the indicator gene viral vector which is then referred to as the resistance test vector, as described above.

"Patient sequence acceptor sites" are sites in a vector for

insertion of patient-derived segments and said sites may be:
1) unique restriction sites introduced by site-directed
mutagenesis into a vector; 2) naturally occurring unique
restriction sites in the vector; or 3) selected sites into
5   which a patient-derived segment may be inserted using
alternative cloning methods (e.g. UDG cloning). In one
embodiment the patient sequence acceptor site is introduced
into the indicator gene viral vector. The patient sequence
acceptor sites are preferably located within or near the
10   coding region of the viral protein which is the target of
the anti-viral drug.   The viral sequences used for the
introduction of patient sequence acceptor sites are
preferably chosen so that no change, or a conservative
change, is made in the amino acid coding sequence found at
15   that position.   Preferably the patient sequence acceptor
sites are located within a relatively conserved region of
the viral genome to facilitate introduction of the
patient-derived segments.   Alternatively, the patient
sequence acceptor sites are located between functionally
20   important genes or regulatory sequences. Patient-sequence
acceptor sites may be located at or near regions in the
viral genome that are relatively conserved to permit priming
by the primer used to introduce the corresponding
restriction site into the patient-derived segment.   To
25   improve the representation of patient-derived segments
further, such primers may be designed as degenerate pools to
accommodate viral sequence heterogeneity, or may incorporate
residues such as deoxyinosine (I) which have multiple
base-pairing capabilities. Sets of resistance test vectors
30   having patient sequence acceptor sites that define the same
or overlapping restriction site intervals may be used
together in the drug resistance and susceptibility tests to
provide representation of patient-derived segments that
contain internal restriction sites identical to a given
35   patient sequence acceptor site, and would thus be

-68-

underrepresented in either resistance test vector alone.

Host Cells

The resistance test vector is introduced into a host cell.
5   Suitable host cells are mammalian cells. Preferred host
cells are derived from human tissues and cells which are the
principle targets of viral infection. In the case of HIV
these include human cells such as human T cells, monocytes,
macrophage, dendritic cells, Langerhans cells, hematopoeitic
10   stem cells or precursor cells, and other cells. In the case
of HBV, suitable host cells include hepatoma cell lines
(HepG2, Huh 7), primary human hepatocytes, mammalian cells
which can be- infected by pseudotyped HBV, and other cells.
Human derived host cells will assure that the anti-viral
15   drug will enter the cell efficiently and be converted by the
cellular enzymatic machinery into the metabolically relevant
form of the anti-viral inhibitor. Host cells are referred
to herein as a "packaging host cells," "resistance test
vector host cells," or "target host cells." A "packaging
20   host cell" refers to a host cell that provides the
trans-acting factors and viral packaging proteins required
by the replication defective viral vectors used herein, such
as the resistance test vectors, to produce resistance test
vector viral particles. The packaging proteins may be
25   provided for by the expression of viral genes contained
within the resistance test vector itself, a packaging
expression vector(s), or both. A packaging host cell is a
host cell which is transfected with one or more packaging
expression vectors and when transfected with a resistance
30   test vector is then referred to herein as a "resistance test
vector host cell" and is sometimes referred to as a
packaging host cell/resistance test vector host cell.
Preferred host cells for use as packaging host cells for HIV
include 293 human embryonic kidney cells (293, Graham, F.L.
35   et al., J. Gen Virol. 36: 59, 1977), BOSC23 (Pear et al.,

Proc. Natl. Acad. Sci. 90, 8392, 1993), tsa54 and tsa201
cell lines (Heinzel et al., *J.Virol*. 62, 3738,1988), for HBV
HepG2 (Galle and Theilmann, L. Arzheim.-Forschy *Drug Res.*
(1990) 40, 1380-1382). (Huh, Ueda, K et al. *Virology* *1989)
5   169, 213-216). A "target host cell" refers to a cell to be
infected by resistance test vector viral particles produced
by the resistance test vector host cell in which expression
or inhibition of the indicator gene takes place. Preferred
host cells for use as target host cells include human T cell
10 leukemia cell lines including Jurkat (ATCC TlB-152), H9
(ATCC HTB-176), CEM (ATCC CCL-119), HUT78 (ATCC TlB-161),
and derivatives thereof.

Drug Susceptibility and Resistance Tests
15 The drug susceptibility and resistance tests of this
invention may be carried out in one or more host cells.
Viral drug susceptibility is determined as the concentration
of the anti-viral agent at which a given percentage of
indicator gene expression is inhibited (e.g. the IC_{50} for an
20 anti-viral agent is the concentration at which 50% of
indicator gene expression is inhibited). A standard curve
for drug susceptibility of a given anti-viral drug can be
developed for a viral segment that is either a standard
laboratory viral segment or from a drug-naive patient (i.e.
25 a patient who has not received any anti-viral drug) using
the method of this invention. Correspondingly, viral drug
resistance is a decrease in viral drug susceptibility for a
given patient either by comparing the drug susceptibility to
such a given standard or by making sequential measurement in
30 the same patient over time, as determined by increased
inhibition of indicator gene expression (i.e. decreased
indicator gene expression).

In the first type of drug susceptibility and resistance
35 test, resistance test vector viral particles are produced by

a first host cell (the resistance test vector host cell) that is prepared by transfecting a packaging host cell with the resistance test vector and packaging expression vector(s). The resistance test vector viral particles are then used to infect a second host cell (the target host cell) in which the expression of the indicator gene is measured. Such a two cell system comprising a packaging host cell which is transfected with a resistance test vector, which is then referred to as a resistance test vector host cell, and a target cell are used in the case of either a functional or non-functional indicator gene. Functional indicator genes are efficiently expressed upon transfection of the packaging host cell and would require infection of a target host cell with resistance test vector host cell supernatant to carry out the test of this invention. Non-functional indicator genes with a permuted promoter, a permuted coding region, or an inverted intron are not efficiently expressed upon transfection of the packaging host cell and thus the infection of the target host cell can be achieved either by co-cultivation by the resistance test vector host cell and the target host cell or through infection of the target host cell using the resistance test vector host cell supernatant. In the second type of drug susceptibility and resistance test, a single host cell (the resistance test vector host cell) also serves as a target host cell. The packaging host cells are transfected and produce resistance test vector viral particles and some of the packaging host cells also become the target of infection by the resistance test vector particles. Drug susceptibility and resistance tests employing a single host cell type are possible with viral resistance test vectors comprising a non-functional indicator gene with a permuted promoter, a permuted coding region, or an inverted intron. Such indicator genes are not efficiently expressed upon transfection of a first cell, but

are only efficiently expressed upon infection of a second
cell, and thus provide an opportunity to measure the effect
of the anti-viral agent under evaluation. In the case of a
drug susceptibility and resistance test using a resistance
5   test vector comprising a functional indicator gene, neither
the co-cultivation procedure nor the resistance and
susceptibility test using a single cell type can be used for
the infection of target cells. A resistance test vector
comprising a functional indicator gene requires a two cell
10   system using filtered supernatants from the resistance test
vector host cells to infect the target host cell.

In one embodiment of the invention in the case of HIV, a
particle-based resistance tests are carried out with
15   resistance test vectors derived from genomic viral vectors,
i.e.,   pLG-lucPP-HS,   pCG-lucPP-HS,   pLG-lucPP-PB,
pCG-lucPP-PB, pLG-lucPC-HS, pCG-lucPC-HS, pLG-lucPC-PB,
pCG-lucPC-PB, pLG-lucII-HS, pCG-lucII-HS, pLG-lucII-PB,
pCG-lucII-PB,   and   pCG-CXCN(F-lucP)2-AA   which   are
20   cotransfected   with   the   packaging   expression   vector
pVL-env4070A   (also   referred   to   as   pCXAS-4070Aenv).
Alternatively, a   particle-based resistance test may be
carried out with resistance test vectors derived from
subgenomic viral vectors, i.e., pLS-lucPP-HS, pCS-lucPP-HS,
25   pLS-lucPP-PB, pCS-luc-PP-PB, pLS-lucPC-HS, pCS-lucPC-HS,
pLS-lucPC-PB, pCS-luc-PC-PB, pLS-lucII-HS, pCS-lucII-HS,
pLS-lucII-PB, and pCS-luc-II-PB) which are cotransfected
with the packaging expression vector pVL-env4070A and either
pLTR-HIV3' or pCMV-HIV3'. In another embodiment of the
30   invention in the case of HIV, non-particle-based resistance
tests are carried out using each of the above described
resistance test vectors by transfection of selected host
cells in the absence of packaging expression vectors.

35   In the case of the particle-based susceptibility and

resistance test, resistance test vector viral particles are produced by a first host cell (the resistance test vector host cell) that is prepared by transfecting a packaging host cell with the resistance test vector and packaging expression vector(s). The resistance test vector viral particles are then used to infect a second host cell (the target host cell) in which the expression of the indicator gene is measured. In a second type of particle-based susceptibility and resistance test, a single host cell type (the resistance test vector host cell) serves both purposes: some of the packaging host cells in a given culture are transfected and produce resistance test vector viral particles and some of the host cells in the same culture are the target of infection by the resistance test vector particles thus produced. Resistance tests employing a single host cell type are possible with resistance test vectors comprising a non-functional indicator gene with a permuted promoter since such indicator genes are efficiently expressed upon infection of a permissive host cell, they are not efficiently expressed upon transfection of the same host cell type, and thus provide an opportunity to measure the effect of the anti-viral agent under evaluation. For similar reasons, resistance tests employing two cell types may be carried out by co-cultivating the two cell types as an alternative to infecting the second cell type with viral particles obtained from the supernatants of the first cell type.

In the case of the non-particle-based susceptibility and resistance test, resistance tests are performed by transfection of a single host cell with the resistance test vector in the absence of packaging expression vectors. Non-particle based resistance tests are carried out using the resistance test vectors comprising non-functional indicator genes with either permuted promoters, permuted

coding regions or inverted introns. These non-particle
based resistance tests are performed by transfection of a
single host cell type with each resistance test vector in
the absence of packaging expression vectors. Although the
non-functional indicator genes contained within these
resistance test vectors are not efficiently expressed upon
transfection of the host cells, there is detectable
indicator gene expression resulting from non-viral
particle-based reverse transcription. Reverse transcription
and strand transfer results in the conversion of the
permuted, non-functional indicator gene to a non-permuted,
functional indicator gene. As reverse transcription is
completely dependent upon the expression of the *pol* gene
contained within each resistance test vector, anti-viral
agents may be tested for their ability to inhibit the *pol*
gene products encoded by the patient-derived segments
contained within the resistance test vectors. In the case
of HIV, reverse transcription and strand transfer results in
the conversion of the non-functional indicator gene to a
functional indicator gene. As reverse transcription is
completely dependent upon the expression of the
patient-derived segment contained within each resistance
test vector, anti-viral agents may be tested for their
ability to inhibit the gene products encoded by the
patient-derived segments contained within the resistance
test vectors.

The packaging host cells are transfected with the resistance
test vector and the appropriate packaging expression
vector(s) to produce resistance test vector host cells.
Individual anti-viral agents, including the reverse
transcriptase inhibitors AZT, ddI, ddC, d4T and 3TC, and the
protease inhibitors saquinavir, ritonavir and indinavir, as
well as combinations thereof, are added to individual plates
of packaging host cells at the time of their transfection,

at an appropriate range of concentrations. Twenty-four to 48
hours after transfection, target host cells are infected by
co-cultivation with resistance test vector host cells or
with resistance test vector viral particles obtained from
5   filtered supernatants of resistance test vector host cells.
Each anti-viral agent, or combination thereof, is added to
the target host cells prior to or at the time of infection
to achieve the same final concentration of the given agent,
or agents, present during the transfection.

10

Determination of the expression or inhibition of the
indicator gene in the target host cells infected by
co-cultivation or with filtered viral supernatants is made
by assay of indicator gene expression, for example in the
15 case where the indicator gene is the firefly *luc* gene, by
measuring luciferase activity. The reduction in luciferase
activity observed for target host cells infected with a
given preparation of resistance test vector viral particles
in the presence of a given antiviral agent, or agents, as
20 compared to a control run in the absence of the antiviral
agent, generally relates to the log of the concentration of
the antiviral agent as a sigmoidal curve. This inhibition
curve is used to calculate the apparent inhibitory
concentration (IC) of that agent, or combination of agents,
25 for the viral target product encoded by the patient-derived
segments present in the resistance test vector.

In the case of a one cell susceptibility and resistance
test, host cells are transfected with the resistance test
30 vector and the appropriate packaging expression vector(s) to
produce resistance test vector host cells. Individual
antiviral agents, or combinations thereof, are added to
individual plates of transfected cells at the time of their
transfection, at an appropriate range of concentrations.
35 Twenty-four to 72 hours after transfection, cells are

-75-

collected and assayed for firefly luciferase activity. As
transfected cells in the culture do not efficiently express
the indicator gene, transfected cells in the culture, as
well superinfected cells in the culture, can serve as target
5   host cells for indicator gene expression. The reduction in
luciferase activity observed for cells transfected in the
presence of a given antiviral agent, or agents as compared
to a control run in the absence of the antiviral agent(s),
generally relates to the log of the concentration of the
10 antiviral agent as a sigmoidal curve. This inhibition curve
is used to calculate the apparent inhibitory concentration
(IC) of an agent, or combination of agents, for the viral
target product encoded by the patient-derived segments
present in the resistance test vector.

15

Antiviral Drugs/Drug Candidates

The antiviral drugs being added to the test system are added
at selected times depending upon the target of the antiviral
drug. For example, in the case of HIV protease inhibitors,
20 including saquinavir, ritonavir, indinavir, and nelfinavir,
they are added to individual plates of packaging host cells
at the time of their transfection with a resistance test
vector, at an appropriate range of concentrations. HIV
protease inhibitors are also added to the target host cells
25 at the time of infection to achieve the same final
concentration added during transfections. HIV reverse
transcriptase inhibitors, including AZT, ddI, ddC, d4T, 3TC
and nevaripine, are added to individual plates of target
host cells at the time of infection by the resistance test
30 vector viral particles, at a test concentration.
Alternatively, the antiviral drugs may be present throughout
the assay. The test concentration is selected from a range
of concentrations which is typically between about 0.1nM and
about 100(M and more specifically for each of the following
35 drugs: AZT, from about 1nM to about 5(M; ddI, from about 1nM

to about 25(M; 3TC, from about 1nM to about 50(M; d4T, from about 1nM to about 25(M; and, nevaripine, from about 1nM to about 100(M.

In another embodiment of this invention, a candidate antiviral compound is tested in the drug susceptibility and resistance test of this invention. The candidate antiviral compound is added to the test system at an appropriate concentration and at selected times depending upon the protein target of the candidate anti-viral. Alternatively, more than one candidate antiviral compound may be tested or a candidate antiviral compound may be tested in combination with an approved antiviral drug such as AZT, ddI, ddC, d4T, 3TC, saquinavir or a compound which is undergoing clinical trials such as ritonavir, or indinovir. The effectiveness of the candidate antiviral will be evaluated by measuring the expression or inhibition of the indicator gene. In another aspect of this embodiment, the drug susceptibility and resistance test may be used to screen for viral mutants. Following the identification of resistant mutants to either known anti-virals or candidate anti-virals the resistant mutants are isolated and the DNA is analyzed. A library of viral resistant mutants can thus be assembled enabling the screening of candidate anti-virals, alone or in combination. This will enable one of ordinary skill to identify effective anti-virals and design effective therapeutic regimens.

General Materials and Methods

Most of the techniques used to construct vectors, and transfect and infect cells, are widely practiced in the art, and most practitioners are familiar with the standard resource materials which describe specific conditions and procedures. However, for convenience, the following paragraphs may serve as a guideline.

"Plasmids" and "vectors" are designated by a lower case p
followed by letters and/or numbers. The starting plasmids
herein are either commercially available, publicly available
on an unrestricted basis, or can be constructed from
available plasmids in accord with published procedures. In
addition, equivalent plasmids to those described are known
in the art and will be apparent to the ordinarily skilled
artisan.

Construction of the vectors of the invention employs
standard ligation and restriction techniques which are well
understood in the art (see Ausubel et al., (1987) Current
Protocols in Molecular Biology, Wiley - Interscience or
Maniatis et al., (1992) in Molecular Cloning: A laboratory
Manual, Cold Spring Harbor Laboratory, N.Y.). Isolated
plasmids, DNA sequences, or synthesized oligonucleotides are
cleaved, tailored, and relegated in the form desired. The
sequences of all DNA constructs incorporating synthetic DNA
were confirmed by DNA sequence analysis (Sanger et al.
(1977) *Proc. Natl. Acad. Sci.* 74, 5463-5467).

"Digestion" of DNA refers to catalytic cleavage of the DNA
with a restriction enzyme that acts only at certain
sequences, restriction sites, in the DNA. The various
restriction enzymes used herein are commercially available
and their reaction conditions, cofactors and other
requirements are known to the ordinarily skilled artisan.
For analytical purposes, typically 1(g of plasmid or DNA
fragment is used with about 2 units of enzyme in about 20(1
of buffer solution. Alternatively, an excess of restriction
enzyme is used to insure complete digestion of the DNA
substrate. Incubation times of about one hour to two hours
at about 37°C are workable, although variations can be
tolerated. After each incubation, protein is removed by
extraction with phenol/chloroform, and may be followed by

-78-

ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in _Methods of Enzymology_ 65:499-560 (1980).

Restriction cleaved fragments may be blunt ended by treating with the large fragment of _E. coli_ DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 minutes at 20°C in 50 mM Tris (ph7.6) 50 mM NaCl, 6 mM $MgCl_2$, 6 mM DTT and 5-10 mM dNTPs. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the dNTPs, or with selected dNTPs, within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or Bal-31 results in hydrolysis of any single-stranded portion.

Ligations are performed in 15-50 (l volumes under the following standard conditions and temperatures: 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 mg/ml BSA, 10 mM-50 mM NaCl, and either 40 mM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1mM ATP, 0.3 - 0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 (g/ml total DNA concentrations (5-100 mM total end concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1 mM total ends concentration.

-79-

"Transient expression" refers to unamplified expression within about one day to two weeks of transfection. The optimal time for transient expression of a particular desired heterologous protein may vary depending on several factors including, for example, any transacting factors which may be employed, translational control mechanisms and the host cell. Transient expression occurs when the particular plasmid that has been transfected functions, i.e., is transcribed and translated. During this time the plasmid DNA which has entered the cell is transferred to the nucleus. The DNA is in a nonintegrated state, free within the nucleus. Transcription of the plasmid taken up by the cell occurs during this period. Following transfection the plasmid DNA may become degraded or diluted by cell division. Random integration within the cell chromatin occurs.

In general, vectors containing promoters and control sequences which are derived from species compatible with the host cell are used with the particular host cell. Promoters suitable for use with prokaryotic hosts illustratively include the beta-lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (trp) promoter system and hybrid promoters such as tac promoter. However, other functional bacterial promoters are suitable. In addition to prokaryotes, eukaryotic microbes such as yeast cultures may also be used. *Saccharomyces cerevisiae,* or common baker's yeast is the most commonly used eukaryotic microorganism, although a number of other strains are commonly available.
Promoters controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, simian virus 40 (SV40), adenovirus, retroviruses, hepatitis B virus and preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. b-actin promoter. The early and late promoters of the SV 40 virus are conveniently obtained

as an SV40 restriction fragment which also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Of course, promoters from the host cell or related species also are useful herein.

The vectors used herein may contain a selection gene, also termed a selectable marker. A selection gene encodes a protein, necessary for the survival or growth of a host cell transformed with the vector. Examples of suitable selectable markers for mammalian cells include the dihydrofolate reductase gene (DHFR), the ornithine decarboxylase gene, the multi-drug resistance gene (mdr), the adenosine deaminase gene, and the glutamine synthase gene. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media. The second category is referred to as dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin (Southern and Berg (1982) *J. Molec. Appl. Genet.* 1, 327), mycophenolic acid (Mulligan and Berg (1980) *Science* 209, 1422), or hygromycin (Sugden et al. (1985) *Mol. Cell. Biol.* 5, 410-413). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug neomycin (G418 or genticin), xgpt (mycophenolic acid) or hygromycin,

-81-

respectively.

"Transfection" means introducing DNA into a host cell so that the DNA is expressed, whether functionally expressed or otherwise; the DNA may also replicate either as an extrachromosomal element or by chromosomal integration. Unless otherwise provided, the method used herein for transformation of the host cells is the calcium phosphate co-precipitation method of Graham and van der Eb (1973) *Virology* 52, 456-457. Alternative methods for transfection are electroporation, the DEAE-dextran method, lipofection and biolistics (Kriegler (1990) Gene Transfer and Expression: A Laboratory Manual, Stockton Press).

Host cells may be transfected with the expression vectors of the present invention and cultured in conventional nutrient media modified as is appropriate for inducing promoters, selecting transformants or amplifying genes. Host cells are cultured in F12:DMEM (Gibco) 50:50 with added glutamine and without antibiotics. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The following examples merely illustrate the best mode now known for practicing the invention, but should not be construed to limit the invention. All literature references are expressly incorporated by reference.

-82-

EXAMPLE 1
HIV Drug Susceptibility And Resistance Test Using Resistance Test Vectors Comprising Patient-derived Segment(s) And A Non-Functional Indicator Gene With A Permuted Promoter.

Indicator Gene Viral Vector - Construction

Indicator gene viral vectors containing a non-functional indicator gene with a permuted promoter were designed using both HIV genomic and subgenomic viral vectors comprising viral genes which are the target(s) of anti-viral drugs. The indicator gene viral vectors pLG-lucPP and pCG-lucPP are based on the genomic viral vectors pLG and pCG; each bears a deletion in the HIV *env* gene. Resistance test vectors derived from the genomic indicator gene viral vectors, pLG-lucPP and pCG-lucPP, contain a patient sequence acceptor site for insertion of the patient-derived segment and are used in conjunction with a packaging expression vector encoding the amphotrophic MLV 4070A *env* gene product. The indicator gene viral vectors pLS-lucPP and pCS-lucPP are based on the subgenomic viral vectors pLS and pCS; each encodes the HIV *gag-pol* gene only. Resistance test vectors derived from the subgenomic indicator gene viral vectors, pLS-lucPP and pCS-lucPP, contain a sequence acceptor site for insertion of patient-derived segment and are used in conjunction with a first packaging expression vector encoding the HIV *vif*, *vpr*, *tat*, *rev*, *vpu* and *nef* genes and a second packaging vector encoding the amphotrophic MLV 4070A *env* gene product.

HIV Viral Vectors - Genomic and Subgenomic

HIV viral vectors were designed using the sequences of the biologically active proviral clone, HXB2 (Fisher et al. (1986) *Nature* 320, 367-371). Two types of viral vector were designed: genomic viral vectors with deletions in a single

gene such as *env*, but which otherwise preserve the mRNA
expression and processing characteristics of the complete
virus, and subgenomic viral vectors which may include only
one or a few genes that are typically the specific targets
of susceptibility and resistance testing, such as *gag-pol*,
or which may lack viral genes altogether. Both types of
vectors have a unique restriction site within the viral
genome for the insertion of an indicator gene cassette as
well as patient sequence acceptor sites, i.e. additional
unique restriction sites near or within the anti-viral
target gene (eg., *pol*) to permit the insertion of
patient-derived HIV sequences. In addition, both types of
vector were designed to incorporate either the native
enhancer-promoter of the HIV-LTR U3 region, or a foreign
enhancer-promoter from the CMV immediate-early (IE) region.
Standard methods are employed for the construction of
plasmid DNAs (Ausubel et al. (1987) Current Protocols in
Molecular Biology, Wiley-Interscience). The sequences of
all DNA constructs incorporating synthetic DNA are confirmed
by DNA sequence analysis (Sanger et al. (1977) *Proc. Natl.
Acad. Sci.* **74**, 5463-5467).

HIV sequences are obtained from plasmid pBS-HIV (Page et al.
(1990) *J. Virol.* **64**, 5270-5276) which contains the HXB2
proviral DNA sequence on an HpaI to XbaI restriction
fragment inserted into the polylinker of the pBluescript KS
(+) plasmid cloning vector (Stratagene, San Diego, CA). As
this proviral clone contains uncharacterized, flanking human
DNA from the site of proviral integration, two steps of
site-directed mutagenesis are employed to remove such
sequences using plasmid pBS-HIV as a template. In step one,
human sequences adjacent to the 5' LTR are removed using
oligonucleotide 1 which contains the following sequences in
a 5' to 3' direction: 1) a sequence complementary to the
first 18 nucleotides of the integrated provirus, within the

left U3 region, 2) a six nucleotide SmaI site, and 3) an 18 nucleotide sequence complementary to the region in pBluescript KS (+) just beyond the polylinker and phage T3 promoter. In step two, human sequences adjacent to the 5' LTR are removed using oligonucleotide 2 which contains the following sequences (5' to 3'): 1) an 18 nucleotide sequence complementary to the region in pBluescript KS (+) just beyond the PvuI site, within the LacZ gene, 2) a six nucleotide XbaI site, and 3) a sequence complementary to the last 18 nucleotides of the integrated provirus, within the right U5 region. The resulting plasmid is called pBS-HXB2.

Genomic and subgenomic viral vectors employing the HIV-LTR U3 region as an enhancer-promoter for the expression of anti-viral target genes (Fig. 1) are each derived from plasmid pBS-HXB2 by a single step of site-directed mutagenesis. The genomic viral vector pLG, which is deleted for the env gene, is prepared using oligonucleotide 3 which contains the following sequences (5' to 3'): 1) an 18 nucleotide sequence complementary to positions 7626 to 7643 of HXB2 within the env gene (all coordinates for HXB2 are by reference to GenBank, accession number K03455), 2) an 8 nucleotide NotI site, and 3) an 18 nucleotide sequence complementary to positions 6384 to 6401 of HXB2 within the env gene. The subgenomic viral vector pLS, which is deleted for the env, tat, rev, vif, vpr and vpu genes, is prepared using oligonucleotide 4 which contains the following sequences (5' to 3'): 1) an 18 nucleotide sequence complementary to positions 7626 to 7643 of HXB2 within the env gene, 2) an 8 nucleotide NotI site, and 3) an 18 nucleotide sequence complementary to positions 5109 to 5126 of HXB2 within the vif gene.

Genomic and subgenomic viral vectors which employ the CMV IE region as an enhancer-promoter for the expression of

anti-viral target genes are derived from plasmids pLG and
pLS, and are called pCG and pCS, respectively (Fig. 1). The
genomic viral vector pCG is prepared in two steps. In the
first step, an intermediate plasmid is prepared from two DNA
fragments: 1) a vector fragment 11.2 kB prepared from
digesting plasmid pLG with SmaI and treating the vector with
alkaline phosphatase, and 2) a DNA fragment of 0.9 kB
containing the CMV IE enhancer-promoter prepared by
digesting plasmid pVL-1 (described below) with SmaI.
Plasmids containing the CMV IE region in the same
transcriptional orientation as the viral LTRs are identified
by restriction mapping. In the second step, plasmid pCG is
prepared from this intermediate plasmid by site-directed
mutagenesis to join the CMV IE enhancer-promoter to the
5'-LTR R region at a position permitting transcription
initiation to occur at the beginning of the R region, using
oligonucleotide 5 which contains the following sequences (5'
to 3'): 1) an 18 nucleotide sequence complementary to
positions 455 to 472 of HXB2 at the beginning of the R
region, and 2) an 18 nucleotide sequence complementary to
positions -18 to -1 of the CMV IE enhancer-promoter
(coordinates referenced to Boshart et al. (1985) *Cell* **41**,
521-530). The subgenomic viral vector pCS is derived from
plasmid pCG and is prepared from two DNA fragments: 1) a
vector DNA of 9.1 kB prepared by digesting plasmid pLS with
SmaI and ClaI, and 2) a DNA fragment of 1.3 kB prepared by
digesting plasmid pCG with SmaI and ClaI.

Genomic Indicator Gene Viral Vector- Permuted Promoter
The indicator gene viral vectors pLG-lucPP and pCG-lucPP,
and resistance test vectors derived therefrom, contain the
following elements in a 5' to 3' orientation (Fig 2B): 1)
an HIV-LTR U3 region (pLG-lucPP) or a CMV IE
enhancer-promoter (pCG-lucPP), 2) an HIV-LTR R region, 3) an
HIV-LTR U5 region containing an inserted T7 promoter with a

transcriptional orientation opposite to that of the LTRs, 4) the coding regions of the HIV *gag-pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, deleted *env*, and *nef* genes, 5) an indicator gene cassette inserted into the deleted *env* gene, and 6) a 3'
HIV-LTR. The same indicator gene cassette is inserted in pLG-lucPP and pCG-lucPP and contains the following elements: 1) an EMC 5'-UTR region which permits internal ribosome entry, 2) the complete coding region of the luciferase (luc) gene, and 3) a T7 transcriptional terminator. The indicator gene has a transcriptional orientation opposite to the HIV-LTR or CMV IE enhancer-promoter and therefore cannot be functionally transcribed by these elements. The indicator gene also cannot be transcribed by the T7 promoter as the indicator gene cassette is positioned upstream of the T7 promoter. Following reverse transcription and strand transfer, the T7 promoter is copied from the 5' LTR to the 3' LTR, permitting functional transcription of the indicator gene from the newly created T7 promoter by T7 RNA polymerase (Fig 2C).

Plasmid plucPP, which contains the indicator gene cassette, is prepared in three steps. In the first step, plasmid pVL-EMC/T7 which contains a cassette flanked by unique NotI sites comprising the EMC 5'-UTR element and T7 transcriptional terminator, is prepared from two DNA fragments: 1) a vector DNA of 3.0 kB prepared by digesting plasmid pVL (described below) with NotI and treating the vector with alkaline phosphatase, and 2) a DNA fragment of 0.8 kB containing the EMC 5' UTR and T7 terminator prepared by PCR using plasmid pTM1 (Moss et al. (1990) *Nature* 348, 91-92) as a template and oligonucleotides 6 and 7 as primers, followed by digestion with NotI. Oligonucleotides 6 and 7 each incorporate a NotI restriction site. In the second step, plasmid pVL-luc, which contains the coding region of the firefly luciferase gene inserted into the

-87-

mammalian expression vector pVL-1 (described below), is prepared from two DNA fragments: a vector DNA of 4.1 kB prepared by digesting plasmid pVL-1 with NruI and BglII, and 2) a DNA fragment of 1.7 kB containing the complete

5   luciferase coding region, prepared by PCR using plasmid pGEM-luc (Promega, Madison, WI) as a template and oligonucleotides 8 and 9 as primers followed by digestion with NruI and BglII. Oligonucleotides 8 and 9 incorporate NruI and NcoI, and BglII and XhoI restriction sites,

10   respectively. In the third step, plasmid plucPP, which contains the coding region of the luciferase gene inserted between the EMC 5'-UTR element and T7 transcriptional terminator, is prepared from two DNA fragments: 1) a vector DNA of 3.8 kB prepared by digesting plasmid pVL-EMC/T7 with

15   NcoI and SalI, and 2) a DNA fragment of 1.7 kB containing the complete luciferase coding region, prepared by digesting plasmid pVL-luc with NcoI and XhoI.

     Plasmid pLG-lucPP is prepared in two steps. In the first

20   step, plasmid pLG-T7 is prepared by inserting a phage T7 promoter into the upstream HIV-LTR U5 region in plasmid pLG by site-directed mutagenesis using oligonucleotide 10 which contains the following sequences (5' to 3'): 1) an 18 nucleotide sequence complementary to positions 552 to 569 of

25   HXB2 within the U5 region, 2) a 20 nucleotide sequence complementary to the T7 promoter, and 3) an 18 nucleotide sequence complementary to positions 534 to 551 of HXB2 within the U5 region. In the second step, plasmid pLG-lucPP is prepared from two DNA fragments: 1) a vector DNA of 11.2

30   kB prepared by digesting plasmid pLG-T7 with NotI and treating the resulting vector with alkaline phosphatase, and 2) a DNA fragment of 2.5 kB containing the luciferase indicator gene cassette prepared by digesting plasmid plucPP with NotI. Clones corresponding to pLG-lucPP, which contain

35   the indicator gene cassette inserted into the viral vector

with a transcriptional orientation opposite to that of the viral LTRs, are identified by restriction mapping.

Plasmid pCG-lucPP is prepared in two steps. In the first step, plasmid pCG-T7 is prepared by inserting a phage T7 promoter into the upstream HIV-LTR U5 region in plasmid pCG by site-directed mutagenesis using oligonucleotide 10. In the second step, plasmid pCG-lucPP is prepared from two DNA fragments: 1) a vector DNA of 11.7 kB prepared by digesting plasmid pCG-T7 with NotI and treating the resulting vector with alkaline phosphatase, and 2) a DNA fragment of 2.5 kB containing the luciferase indicator gene cassette prepared by digesting plasmid plucPP with NotI. Clones corresponding to pCG-lucPP, which contain the indicator gene cassette inserted into the viral vector with a transcriptional orientation opposite to that of the CMV IE enhancer-promoter and viral LTRs, are identified by restriction mapping.

Subgenomic Indicator Gene Viral Vector - Permuted Promoter
The indicator gene viral vectors pLS-lucPP and pCS-lucPP, and resistance test vectors derived therefrom, contain the following elements in a 5' to 3' orientation (Fig 2B): 1) an HIV-LTR U3 region (pLS-lucPP) or a CMV IE enhancer-promoter (pCS-lucPP), 2) an HIV-LTR R region, 3) an HIV-LTR U5 region containing an inserted T7 promoter with a transcriptional orientation opposite to that of the LTRs, 4) the coding region of the HIV *gag-pol* gene, 5) the indicator gene cassette, 6) an RRE element from the HIV *env* gene containing a viral packaging sequence, and 7) a 3' HIV- LTR. The indicator gene cassette of pLS-lucPP and pCS-lucPP is the same as in pLG-lucPP and pCG-lucPP. As for the latter vectors, the indicator genes of pLS-lucPP and pCS-lucPP cannot be functionally transcribed until reverse transcription and strand transfer results in the copying of the T7 promoter from the 5' LTR to the 3' LTR (Fig 2C).

Plasmid pLS-lucPP is prepared in two steps. In the first step, plasmid pLS-T7, which contains a phage T7 promoter inserted into the upstream HIV-LTR U5 region of plasmid pLS, is prepared from two DNA fragments: 1) a vector DNA of 9.1 kB prepared by digesting plasmid pLS with SmaI and ClaI, and 2) a DNA fragment of 0.8 containing the HIV-LTR with an R5 region containing an inserted T7 promoter, prepared by digesting plasmid pLG-T7 with SmaI and ClaI. In the second step, plasmid pLS-lucPP is prepared from two DNA fragments: 1) a vector DNA of 9.9 kB prepared by digesting plasmid pLS-T7 with NotI and treating the resulting vector with alkaline phosphatase, and 2) a DNA fragment of 2.5 kB containing the luciferase indicator gene cassette prepared by digesting plasmid plucPP with NotI. Clones corresponding to pLS-lucPP, which contain the indicator gene cassette inserted into the viral vector with a transcriptional orientation opposite to that of the viral LTRs, are identified by restriction mapping.

Plasmid pCS-lucPP is prepared from two DNA fragments: 1) a vector DNA of 11.6 kB prepared by digesting plasmid pLS-lucPP with SmaI and ClaI, and 2) a DNA fragment of 1.3 containing the CMV IE promoter fused to the R5 region with an inserted T7 promoter, prepared by digesting plasmid pCG-T7 with SmaI and ClaI.

Resistance Test Vectors - Construction
Resistance test vectors are prepared by 1) modifying the indicator gene viral vectors pLG-lucPP, pCG-lucPP, pLS-lucPP and pCS-lucPP by introducing unique restriction sites, called patient sequence acceptor sites, in or near the *pol* gene, 2) amplifying patient-derived segments corresponding to the HIV protease and reverse transcriptase coding regions by PCR using complementary DNA (cDNA) prepared from viral RNA or DNA present in the serum or cells of infected

patients, and 3) inserting the amplified sequences precisely
into indicator gene viral vectors at patient sequence
acceptor sites (Fig. 2B). Two sets of patient sequence
acceptor sites are introduced by site-directed mutagenesis
5   into each of the four indicator gene viral vectors. The
first set of patient sequence acceptor sites consist of a
HpaI site and a SalI site which define an interval
comprising the entire protease coding region and most of the
reverse transcriptase coding region, resulting in plasmids
10   pLG-lucPP-HS, pCG-lucPP-HS, pLS-lucPP-HS and pCS-lucPP-HS.
The second set of patient sequence acceptor sites consist of
a PvuI site and a BamHI site which define the same interval,
resulting in plasmids pLG-lucPP-PB, pCG-lucPP-PB,
pLS-lucPP-PB and pCS-lucPP-PB, respectively. Cognate pairs
15   of resistance test vectors which define the same restriction
site interval (eg., those derived from pLG-lucPP-HS and
pLG-lucPP-PB) are used together in some resistance tests to
improve the representation of those patient-derived segments
that contain internal restriction sites identical to a given
20   patient sequence acceptor site, and would thus be
underrepresented in either resistance test vector alone.

Plasmid pLG-lucPP-HS is prepared by three consecutive steps
of site-directed mutagenesis using plasmid pLG-lucPP as a
25   template. The first two steps are for the purpose of
introducing two new restriction sites, one of which (HpaI)
is unique to, and one of which (SalI) is already is present
once in each indicator gene viral vector. The third step is
for the purpose of deleting the pre-existing SalI site in
30   each vector to make the introduced SalI site unique. In
step 1, a HpaI site is introduced immediately upstream of
the mature coding region of the HIV protease at position
2243 using oligonucleotide 11 which contains the following
sequences (5' to 3'): 1) an 18 nucleotide sequence
35   complementary to positions 2249 to 2266 of HXB2, 2) a six

nucleotide HpaI site, which leaves the *gag* protein sequence at this position unaltered and introduces a conservative amino acid change (Phe to Val) into the *pol* precursor sequence, and 3) an 18 nucleotide sequence complementary to positions 2225 to 2242 of HXB2. In step 2, a SalI site is introduced at the carboxy-terminal coding region of the HIV reverse transcriptase at position 4190 using oligonucleotide 12 which contains the following sequences (5' to 3'): 1) an 18 nucleotide sequence complementary to positions 4196 to 4213 of HXB2, 2) a six nucleotide SalI site which leaves the reverse transcriptase protein sequence at this position unaltered, and 3) an 18 nucleotide sequence complementary to positions 4172 to 4189 of HXB2. In step 3, the pre-existing SalI site within the *vpr* coding region at position 5785 of HXB2 is deleted using oligonucleotide 13 which contains the following sequences (5' to 3'): 1) an 18 nucleotide sequence complementary to positions 5791 to 5808 of HXB2, 2) the 6 nucleotide sequence GCCGAC which ablates the SalI site but leaves the *vpr* protein sequence at this position unaltered, 3) an 18 nucleotide sequence complementary to positions 5767 to 5784 of HXB2.

Plasmids pCG-lucPP-HS, pLS-lucPP-HS and pCS-lucPP-HS are derived from pLG-lucPP-HS as follows. Plasmid pCG-lucPP-HS is prepared from two DNA fragments: 1) a vector DNA of 12.9 kB prepared by digesting plasmid pLG-lucPP-HS with SmaI and ClaI, and 2) a DNA fragment of 1.3 kB prepared by digesting plasmid pCG-lucPP with SmaI and ClaI. Plasmid pLS-lucPP-HS is prepared from two DNA fragments: 1) a vector DNA of 11.1 kB prepared by digesting plasmid pLG-lucPP-HS with NdeI and XhoI, and 2) a DNA fragment of 1.3 kB prepared by digesting plasmid pLS-lucPP with NdeI and XhoI. Plasmid pCS-lucPP-HS is prepared from two DNA fragments: 1) a vector DNA of 11.6 kB prepared by digesting plasmid pLS-lucPP-HS with SmaI and ClaI, and 2) a DNA fragment of 1.3 kB prepared by digesting

plasmid pCS-lucPP with SmaI and ClaI.

Plasmid pLG-lucPP-PB is prepared by four consecutive steps
of site-directed mutagenesis using plasmid pLG-lucPP as a
template. The first two steps are for the purpose of
introducing two new restriction sites (PvuI and BamHI), each
of which is already present once in each indicator gene
viral vector. The third and fourth steps are for the
purpose of deleting the pre-existing PvuI and BamHI sites in
each vector to make the newly introduced sites unique. In
step 1, a PvuI site is introduced immediately upstream of
the mature coding region of the HIV protease at position
2221 using oligonucleotide 14 which contains the following
sequences (5′ to 3′): 1) an 18 nucleotide sequence
complementary to positions 2227 to 2244 of HXB2, 2) a six
nucleotide PvuI site, which leaves the *gag* and *pol* precursor
protein sequences at this position unaltered, and 3) an 18
nucleotide sequence complementary to positions 2203 to 2220
of HXB2. In step 2, a BamHI site is introduced at the
carboxy-terminal coding region of the HIV reverse
transcriptase at position 4212 using oligonucleotide 15
which contains the following sequences (5′ to 3′): 1) an 18
nucleotide sequence complementary to positions 4218 to 4235
of HXB2, 2) a six nucleotide BamHI site which leaves the
reverse transcriptase protein sequence at this position
unaltered, and 3) an 18 nucleotide sequence complementary to
positions 4194 to 4211 of HXB2. In step 3, the pre-existing
PvuI site within the b-lactamase coding region at position
2413 (coordinates references to GenBank, accession number
X52331) is deleted using oligonucleotide 16 which contains
the following sequences (5′ to 3′): 1) an 18 nucleotide
sequence complementary to positions 2395 to 2412 of
pBluescript KS (+) , 2) the 6 nucleotide sequence CAATCG
which ablates the PvuI site but leaves the b-lactamase
protein sequence at this position unaltered, and 3) an 18

nucleotide sequence complementary to positions 2419 to 2436
of pBluescript KS (+). In step 4, the pre-existing BamHI
site within the HIV *rev* coding region at position 8474 of
HXB2 is deleted using oligonucleotide 17 which contains the
5   following sequences (5' to 3'): 1) an 18 nucleotide sequence
complementary to positions 8480 to 8497 of HXB2, 2) the 6
nucleotide sequence GGATTC which ablates the BamHI site but
leaves the HIV *rev* protein sequence at this position
unaltered, and 3) an 18 nucleotide sequence complementary to
10   positions 8456 to 8473 of HXB2.

Plasmids pCG-lucPP-PB, pLS-lucPP-PB and pCS-lucPP-PB are
derived from pLG-lucPP-PB as follows. Plasmid pCG-lucPP-PB
is prepared from two DNA fragments: 1) a vector DNA of 12.9
15   kB prepared by digesting plasmid pLG-lucPP-PB with SmaI and
ClaI, and 2) a DNA fragment of 1.3 kB prepared by digesting
plasmid pCG-lucPP with SmaI and ClaI. Plasmid pLS-lucPP-PB
is prepared from three DNA fragments: 1) a vector DNA of
11.1 kB prepared by digesting plasmid pLG-lucPP-PB with NdeI
20   and XhoI, 2) a DNA fragment of 0.5 kB prepared by digesting
plasmid pLS-lucPP with NdeI and HindIII and 3) a DNA
fragment of 0.8 kB prepared by digesting plasmid
pLG-lucPP-PB with HindIII and XhoI. Plasmid pCS-lucPP-PB is
prepared from two DNA fragments: 1) a vector DNA of 11.6 kB
25   prepared by digesting plasmid pLS-lucPP-PB with SmaI and
ClaI, and 2) a DNA fragment of 1.3 kB prepared by digesting
plasmid pCS-lucPP with SmaI and ClaI.

Patient-derived segment(s) corresponding to the HIV protease
30   and reverse transcriptase coding regions are amplified by
the reverse transcription-polymerase chain reaction method
(RT-PCR), using viral RNA isolated from the serum of
HIV-infected patients. Two RT-PCR protocols are used as
described. In the first method (Piatak et al. (1993)
35   *Science* 259, 1749-1754), separate enzymes, Moloney murine

leukemia virus reverse transcriptase (BRL, Bethesda, MD) and *Taq* DNA polymerase (Roche Molecular Diagnostics, Ontario, Canada), are used for the preparation of cDNA and for the PCR reaction, respectively. In the second method (Mulder et al. (1994) J. Clin. Microbiol. 32, 292-300), a single enzyme, *Thermus thermophilus* (*Tth*) DNA polymerase, is used to carry both cDNA synthesis and the PCR reaction. Two primer pairs, consisting of oligonucleotides 18 and 19, and oligonucleotides 20 and 21, are employed for the amplification of patient-derived segments that can be inserted precisely into the indicator gene viral vectors containing the HpaI/SalI and PvuI/BamHI patient acceptor sites, respectively.

A first set of four resistance test vectors incorporating the first primer pair is constructed from the following two DNA preparations: 1) a vector DNA prepared from plasmid pLG-lucPP-HS, pCG-lucPP-HS, pLS-lucPP-HS or pCS-lucPP-HS, digested with HpaI and SalI, and 2) an amplified DNA product of 2.0 kB prepared by RT-PCR using viral RNA isolated from the serum of an HIV-infected individual as a template and oligonucleotides 18 and 19 as primers, followed by digestion with HpaI and SalI. A second set of four resistance test vectors incorporating the second primer pair are constructed from the following two DNA preparations: 1) a vector DNA prepared from plasmid pLG-lucPP-PB, pCG-lucPP-PB, pLS-lucPP-PB or pCS-lucPP-PB, digested with PvuI and BamHI, and 2) an amplified DNA product of 2.0 kB prepared by RT-PCR using viral RNA isolated from the serum of an HIV-infected individual as a template and oligonucleotides 18 and 19 as primers, followed by digestion with PvuI and BamHI. Oligonucleotides 18, 19, 20 and 21 incorporate HpaI, SalI, PvuI and BamHI restriction sites, respectively. To ensure that the plasmid DNA corresponding to each of the eight resulting resistance test vectors comprises a representative

-95-

sample of the HIV viral quasi-species present in the serum of a given patient, at least one hundred independent E. coli transformants obtained in the construction of a given resistance test vector are used for the preparation of plasmid DNA.

To improve the representation of patient-derived segments, a third and fourth set of resistance test vectors are prepared using partially degenerate PCR primer pools, called oligonucleotides 22, 23, 24 and 25, which are based on the sequences of oligonucleotides 18, 19, 20 and 21, respectively. Each primer pool is synthesized in a manner that incorporates more than one nucleotide base (G, A, T or C) at the each of the 18 nucleotide positions located at the 3' end of the parent primer that display sequence variations among the different patient isolates cataloged in the Los Alamos HIV sequence database (Myers et al. (1993) Human Retroviruses and AIDS 1993, Los Alamos National Laboratory, Los Alamos, NM). The third set of four resistance test vectors is constructed using vectors prepared from plasmid pLG-lucPP-HS, pCG-lucPP-HS, pLS-lucPP-HS or pCS-lucPP-HS, with amplified patient sequences prepared with oligonucleotides 22 and 23; the fourth set of four resistance test vectors is constructed using vectors prepared from plasmid pLG-lucPP-PB, pCG-lucPP-PB, pLS-lucPP-PB or pCS-lucPP-PB, with amplified patient sequences prepared with oligonucleotides 24 and 25. Oligonucleotides 22, 23, 24 and 25 incorporate HpaI, SalI, PvuI and BamHI restriction sites, respectively.

Host Cells - Preparation
Packaging Host Cells and Resistance Test Vector Host Cells

Resistance test vectors are used to prepare resistance test vector host cells from packaging host cells expressing viral

packaging proteins. The packaging proteins may be provided
for by the expression of viral genes contained within the
resistance test vector itself, a packaging expression
vector(s), or both. Either transient or stable transfection

5   of the packaging host cell may be employed to produce the
packaging proteins. A packaging expression vector encoding
an amphotrophic MLV *env* gene product enables production in
a resistance test vector host cell of resistance test vector
viral particles which can efficiently infect human target

10   cells.   Resistance test vectors derived from plasmids
pLG-lucPP-HS, pLG-lucPP-PB, pCG-lucPP-HS and pCG-lucPP-PB
encode all HIV genes with the exception of *env*, and are used
to produce resistance test vector host cells.   The
pVL-env4070A packaging expression vector which encodes the

15   amphotrophic MLV 4070A *env* gene product is used with the
foregoing genomic-based resistance test vectors to enable
production in the resistance test vector host cell of
resistance test vector viral particles.   Resistance test
vectors derived from plasmids pLS-lucPP-HS, pLS-lucPP-PB,

20   pCS-lucPP-HS and pCS-lucPP-PB encode the HIV *gag-pol* gene
products only, and are used to prepare resistance test
vector host cells. The pVL-env4070A which provides *env*, and
either the pLTR-HIV3' or the pCMV-HIV3' packaging expression
vectors, each of which provides the HIV *vif*, *vpr*, *tat*, *rev*,

25   *vpu* and *nef* genes are used with the foregoing subgenomic
based resistance test vectors to enable production in the
resistance test vector host cells of resistance test vector
viral particles.

30   Plasmids pLTR-HIV3' and pCMV-HIV3' are each derived by
removing most of the *gag-pol* coding region from the genomic
viral vectors pLG and pCG, respectively. Plasmid pLTR-HIV3'
(Fig. 3B) is prepared by site-directed mutagenesis using
plasmid pLG as a template with oligonucleotide 26 which

35   contains the following sequences (5' to 3'): 1) an 18

-97-

nucleotide sequence complementary to positions 4712 to 4729 of HXB2 within the *pol* gene, and 2) an 18 nucleotide sequence complementary to positions 925 to 942 of HXB2 within the *gag* gene. Plasmid pCMV-HIV3' (Fig. 3C) is

5 prepared from two DNA fragments: 1) a vector fragment of 6.8 kB prepared by digesting plasmid pLTR-HIV3' with SmaI and ClaI, and 2) a DNA fragment of 1.3 kB prepared by digesting plasmid pCG with SmaI and ClaI.

10 Plasmid pVL-env4070A (Fig. 3D) is constructed from two DNA fragments: 1) a vector fragment of 4.3 kB prepared by digestion of the pVL-2 mammalian expression vector with NruI and BglII, and 2) a DNA fragment of 2.0 kB containing the complete coding region of the MLV4070A *env* gene product

15 (nucleotides 37 to 2001, coordinates given in GenBank, accession number M33469, Ott et al. (1990) *J. Virol.* **64**, 757-766) prepared by PCR using plasmid pCRIPam*gag*-2 (Danos and Mulligan (1988) *Proc. Natl. Acad. Sci.* **85**, 6460) as a template with oligonucleotides 27 and 28 as primers,

20 followed by digestion with NruI and BglII. Oligonucleotide 27 incorporates a unique NruI site followed by a consensus sequence for mammalian translation initiation (e.g., Kozak (1991) *J. Biol. Chem,* **266**, 19867-19870); while oligonucleotide 28 incorporates a unique BglII site.

25

The mammalian expression vector pVL-2 contains the following elements in a 5' to 3' direction: the CMV IE promoter/enhancer, the CMV IE first exon splice donor, the human (1 globin second exon splice acceptor, a cloning site

30 polylinker, the polyadenylation site of the SV40 T antigen gene, and the SV40 origin of replication. Plasmid pVL-2 is constructed in four steps as follows. In the first step, plasmid pVL is prepared by replacing the cloning site polylinker and phage T7 and T3 promoters of plasmid

35 pBluescript II KS (+) with a cloning site polylinker

BEST AVAILABLE COPY

containing BssHII, NotI, SmaI, HindIII, SphI, SmaI, EcoRI, NruI, ApaI, BglII, NheI, NotI, XhoI, and BssHII restriction sites. pVL is constructed from two DNA fragments: 1) a vector fragment of 3.0 kB prepared by cutting plasmid pBluescript II KS (+) with BssHII, and treating the resulting vector with alkaline phosphatase, and 2) a DNA fragment prepared by annealing overlapping oligonucleotides 29 and 30, extending with Klenow DNA polymerase and digesting with BssHII. Plasmids containing the HindIII to XhoI sites in a 5' to 3' order relative to the pBluescript II KS (+) plasmid map (GenBank accession number X52327) are identified by restriction mapping analysis. In the second step, an intermediate plasmid is prepared from plasmid pVL by inserting the CMV IE enhancer-promoter and first exon splice donor, and the human (1 globin second exon splice acceptor. This intermediate plasmid is prepared from three DNA fragments: 1) a vector fragment of 3.0 kB prepared by digesting plasmid pVL with HindIII and EcoRI, 2) a DNA fragment of 0.9 kB containing a CMV IE promoter-enhancer and first exon splice donor (nucleotides -674 to -19, coordinates referenced to Boshart et al. (1985) *Cell* **41**, 521-530), prepared by PCR using the plasmid pCM5027 containing the PstI m-fragment from HCMV strain AD169 (Boshart et al., Ibid) as template with oligonucleotides 31 and 32 as primers, followed by digestion with HindIII and SphI, and 3) a DNA fragment of 0.1 kB containing the human (1 globin second exon splice acceptor (nucleotides 6808 to 6916, coordinates by reference to GenBank, accession number J00153) prepared by PCR using plasmid ppSVaHP (Treisman et al. (1983) *Proc. Natl. Acad. Sci.* **80**, 7428-7432) as a template with oligonucleotides 33 and 34 as primers, followed by digestion with SphI and EcoRI. Oligonucleotides 31, 32, 33 and 34 incorporate HindIII, SphI, SphI and EcoRI restriction sites at their respective ends. In the third step, plasmid pVL-1 is prepared by inserting the SV40 T

antigen polyadenylation site into this intermediate plasmid.
Plasmid pVL-1 is prepared from two DNA fragments: 1) a
vector fragment of 4.0 kB prepared by cutting the
intermediate plasmid with BglII and NheI, and 2) a DNA

5   fragment of 0.2 kB containing the SV40 T antigen
polyadenylation site (nucleotides 2770 to 2533 of SV40,
coordinates by reference to Reddy et al. (1978) *Science* **200**,
494-502), prepared by PCR using plasmid pSV2 (Southern and
Berg (1982) *J. Mol. Appl. Gen.* **1**, 327-341) as template with

10   oligonucleotides 35 and 36 as primers, followed by digestion
with BglII and NheI. Oligonucleotides 35 and 36 incorporate
unique BglII and NheI restriction sites at their respective
ends. In the fourth step, plasmid pVL-2 is prepared by
inserting the SV40 origin of replication into plasmid pVL-1.

15   Plasmid pVL-2 is prepared from two DNA fragments: 1) a
vector fragment of 4.2 kB prepared by digesting plasmid
pVL-1 with NheI and XhoI, and 2) a DNA fragment of 0.2 kB
containing the SV40 origin of replication (nucleotides 5725
to 5578 of SV40, Ibid) prepared by PCR using plasmid pSV2 as

20   template with oligonucleotides 37 and 38 as primers,
followed by digestion with NheI and SalI. Oligonucleotides
37 and 38 incorporate unique NheI and SalI restriction sites
at their respective ends.

25

Target Host Cells

Target host cells used for resistance tests carried out with
resistance test vectors derived from plasmids pLG-lucPP-HS,

30   pLG-lucPP-PB, pCG-lucPP-HS, pCG-lucPP-PB, pLS-lucPP-HS,
pLS-lucPP-PB, pCS-lucPP-HS or pCS-lucPP-PB are prepared from
the human embryonic kidney cell line 293 and the Jurkat
leukemic T cell line (American Type Culture Collection,
Rockville, MD). Each cell line is stably transfected with

35   an expression vector encoding a variant phage T7 RNA

BEST AVAILABLE COPY

-100-

polymerase. This variant contains an SV40 T antigen nuclear localization signal (NLS) fused in frame to the N-terminus of the T7 RNA polymerase, permitting its transport into, and function in, the cell nucleus (Lieber et al. (1989) *Nucleic*

5   *Acids Res.* 17, 8485-8493). The non-functional indicator gene in the resistance test vector is converted into a functional indicator gene by reverse transcriptase upon infection of the target cells, resulting from the repositioning of the T7 promoter relative to the indicator

10 gene coding region. Following the integration of the repaired indicator gene into the target cell chromosome by HIV integrase, the nuclear T7 RNA polymerase expressed by the target cell is capable of functionally transcribing the indicator gene.

15

Plasmid pVL-T7RNAP-NLS is used to direct the expression of a variant T7 RNA polymerase linked to an NLS, in human and other mammalian cells and cell lines. pVL-T7RNAP-NLS is prepared from three DNA fragments: 1) a vector fragment of

20 4.3 kB prepared by digestion of the plasmid pVL-2 with EcoRI and BglII, 2) a DNA fragment of 2.6 kB encoding T7 RNA polymerase amino acid residues 34 to 883 (nucleotides 267 to 2817, coordinates by reference to GenBank, accession number M38308, Grachev and Pletnev (1984) *Bioorg. Khim.* 10,

25 824-843) prepared by PCR using plasmid pT7-G1 (Deng et al. (1994) *Gene* 143, 245-249) as a template with oligonucleotides 39 and 40 as primers, followed by digestion with NruI and BglII, and 3) a synthetic DNA fragment encoding the first three amino acids of SV40 T antigen

30 followed by amino acids 118 to 133 of the SV40 large T antigen containing the NLS (Lieber et al., Ibid), prepared by annealing the overlapping oligonucleotides 41 and 42, extending with Klenow DNA polymerase and digesting with EcoRI and NruI. Oligonucleotides 39, 40, 41, 42 incorporate

35 NruI, BglII, EcoRI and NruI restrictions site, respectively.

Plasmid pVL-Neo is employed as a selectable marker for the establishment of stable transfectants of human and other mammalian cells and cell lines by co-transfection. pVL-Neo directs the expression of neomycin phosphotransferase and

5 confers resistance to the antibiotic G418. Plasmid pVL-Neo is prepared from two DNA fragments: 1) a vector fragment of 4.3 kB prepared by digesting plasmid pVL-2 with EcoRI and BglII, and 2) a DNA fragment of 0.8 kB containing the complete Neo coding region (nucleotides 1551 to 2345 of the

10 Tn5 transposon sequence, coordinates given in GenBank accession number U00004, Beck et al. (1982) *Gene* **19**, 327-336) prepared by PCR using plasmid pSV2neo (Southern and Berg (1982) *J. Mol. Appl. Gen.* **1**, 327-341) as a template with oligonucleotides 43 and 44 as primers, followed by

15 digestion with EcoRI and BglII. Oligonucleotide 43 incorporates a unique EcoRI site followed by a consensus sequence for mammalian translation initiation, while oligonucleotide 44 incorporates a unique BglII site.

20 pVL-T7RNAP is introduced by stable transfection into 293 cells by the calcium phosphate coprecipitation method (Wigler et al. (1979) *Cell* **16**, 777) and into Jurkat cells by electroporation (Irving et al. (1991) *Cell* **64**, 891-901). 293 cells are maintained in DMEM medium (JRH Biosciences)

25 supplemented with 1 g/L glucose, 10% donor calf serum (Tissue Culture Biologics). Jurkat cells are maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Irvin Scientific), glutamine, penicillin and streptomycin. Transfection cocktails for 293 cells and Jurkat cells each

30 contain a mixture of 10 micrograms of pVL-T7RNAP and the selectable marker pVL-Neo in a mass ratio of 10:1 to 20:1. Twenty-four to 48 hours following transfection, cells are replated in the same media containing the antibiotic G418 (GIBCO, Grand Island, N.Y.). Independent 293 cell clones

35 resistant to G418 are picked directly from selection plates

after two weeks and are expanded for analysis. Independent Jurkat cell clones resistant to G418 are obtained by limiting dilution after two to three weeks of drug selection and are expanded for analysis.

G418-resistant 293 and Jurkat cell clones are screened for their level of expression of T7 RNA polymerase by determining the level of steady-state T7 RNA polymerase-specific mRNA synthesized by the cells using the Northern blot hybridization method (Ausubel et al. (1987) Current Protocols in Molecular Biology, Wiley-Interscience). 293 and Jurkat cell clones expressing optimal levels of the T7 RNA polymerase are then identified by determining their ability to support T7 RNA polymerase-specific transcription in transient transfections with plasmid pEMCLucbgAn (Deng et al. (1991) Gene 109, 193-201) in which the transcription of the luciferase gene is directed by a T7 promoter. 293 and Jurkat clones supporting the highest levels of luciferase gene expression are chosen for further use; these are referred to as 293/T7RNAP-NLS cells and Jurkat/T7RNAP-NLS cells, respectively.

Drug Susceptibility and Resistance Tests

Resistance tests are carried out with resistance test vectors based on indicator gene viral vectors pLG-lucPP-HS, pLG-lucPP-PB, pCG-lucPP-HS, pCG-lucPP-PB, pLS-lucPP-HS, pLS-lucPP-PB, pCS-lucPP-HS or pCS-lucPP-PB, using either two types of host cell or one type of host cell. In the first type of resistance test, resistance test vector viral particles are produced by a first host cell (the resistance test vector host cell) that is prepared by transfecting a packaging host cell with the resistance test vector and packaging expression vector(s). The resistance test vector viral particles are then used to infect a second host cell (the target host cell) in which the expression of the

indicator gene is measured. In the second type of
resistance test, a single host cell type (the resistance
test vector host cell) serves both purposes: some of the
packaging host cells in a given culture are transfected and
5   produce resistance test vector viral particles and some of
the host cells in the same culture are the target of
infection by the resistance test vector particles thus
produced. Resistance tests employing a single host cell
type are possible with resistance test vectors comprising a
10   non-functional indicator gene with a permuted promoter:
while such indicator genes are efficiently expressed upon
infection of a permissive host cell, they are not
efficiently expressed upon transfection of the same host
cell type, and thus provide an opportunity to measure the
15   effect of the anti-viral agent under evaluation. For
similar reasons, resistance tests employing two cell types
may be carried out by co-cultivating the two cell types as
an alternative to infecting the second cell type with viral
particles obtained from the supernatants of the first cell
20   type.

Susceptibility and Resistance Test - Two Cell
Resistance test vector host cells are prepared by the
cotransfection of a resistance test vector and the
25   appropriate packaging expression vector(s) using either the
293 cell line, the tsa54 or tsa201 cell lines (Heinzel et
al. (1988) J. Virol. 62, 3738), or the BOSC 23 cell line
(Pear et al. (1993) Proc. Natl. Acad. Sci. 90, 8392) as
packaging host cells. Resistance test vectors constructed
30   by inserting patient-derived segment into pLG-lucPP-HS,
pCG-lucPP-HS,   pLG-lucPP-PB   and   pCG-lucPP-PB   are
cotransfected with the packaging expression vector
pVL-env4070A, while resistance test vectors prepared by
inserting patient-derived segments into pLS-lucPP-HS,
35   pCS-lucPP-HS,   pLS-lucPP-PB   and   pCS-lucPP-PB   are

cotransfected with the packaging expression vectors pVL-env4070A and either pLTR-HIV3' or pCMV-HIV3'. Jurkat/T7RNAP-NLS cells are employed as target host cells.

Packaging host cells are grown in DMEM media, 1 g/L glucose, 10% donor calf serum and passaged at a 1:10 dilution every three days. Cells are plated 48 hours prior to transfection at 1x10⁶ cells per 10 cm plate. Cells are transfected by the calcium phosphate coprecipitation method using 5 to 10 mg each of the resistance test vector and the appropriate packaging expression vector(s) to produce resistance test vector host cells. Individual anti-viral agents, including the reverse transcriptase inhibitors AZT, ddI, ddC, d4T and 3TC, and the protease inhibitors saquinavir, ritonavir and indinavir, as well as combinations thereof, are added to individual plates of transfected cells at the time of their transfection, at an appropriate range of concentrations. Twenty-four to 48 hours after transfection, target host cells are infected by co-cultivation with resistance test vector host cells or with viral particles obtained from filtered supernatants of resistance test vector host cells. Each anti-viral agent, or combination thereof, is added to the target host cells at the time of infection to achieve the same final concentration of the given agent, or agents, present during the transfection.

For infection by co-cultivation, media is removed from a 10 cm plate of resistance test vector host cells prepared by transfection 24 to 48 hours earlier, and 0.5 to 1.0 x 10⁶ Jurkat/T7RNAP-NLS target cells are added to the plate in Jurkat cell media containing the anti-viral agent at the appropriate concentration. Target cells are co-cultivated with the resistance test vector host cells for 24 hours, then removed and added to freshly prepared resistance test vector host cells for a second co-cultivation in Jurkat cell

-105-

media containing the anti-viral agent(s) at the appropriate concentration. Twenty-four hours later, the target host cells are harvested from the second co-cultivation, collected by centrifugation, washed three times with
5   ice-cold phosphate-buffered saline (PBS), and assayed for luciferase activity. For infection with filtered supernatants, media is removed from a 10 cm plate of resistance test vector host cells prepared by transfection 24 to 48 hours earlier. The media is filtered through a
10   0.45 mm filter at the time of harvest, frozen at -70°C, and thawed before transduction. Jurkat/T7RNAP-NLS cells (0.5 to 1.0 x 10^6) are added to 5 ml of an equal mixture of Jurkat cell media and the filtered supernatant, made up to 8 mg/ml of polybrene (Sigma, St. Louis, MI) and the appropriate
15   concentration of the anti-viral agent(s). Twenty-four to 48 hours post-infection, the target host cells are collected by centrifugation, washed three times with ice-cold phosphate-buffered saline, and assayed for indicator gene expression. Target host cells infected by co-cultivation or
20   with filtered viral supernatants are assayed for firefly luciferase activity as described (Ausubel et al. (1987) Current Protocols in Molecular Biology, Wiley-Interscience). The reduction in luciferase activity observed for target host cells infected with a given preparation of resistance
25   test vector viral particles in the presence of a given anti-viral agent, or agents, as compared to a control run in the absence of the anti-viral agent, generally relates to the log of the concentration of the anti-viral agent as a sigmoidal curve. This inhibition curve is used to calculate
30   the apparent inhibitory concentration (IC) of that agent, or combination of agents, for the viral target product encoded by the patient-derived segments present in the resistance test vector.

<u>Susceptibility and Resistance Test - One Cell</u>
Resistance test vector host cells are prepared by the
cotransfection of a resistance test vector and the
appropriate packaging expression vector(s) using either
5   293/T7RNAP-NLS cells or Jurkat/T7RNAP-NLS cells as packaging
host cells. Resistance test vectors constructed by
inserting patient-derived segments into pLG-lucPP-HS,
pCG-lucPP-HS, pLG-lucPP-PB and pCG-lucPP-PB are
cotransfected with the packaging expression vector
10   pVL-env4070A, while resistance test vectors prepared by
inserting patient-derived segments into pLS-lucPP-HS,
pCS-lucPP-HS, pLS-lucPP-PB and pCS-lucPP-PB are
cotransfected with the packaging expression vectors
pVL-env4070A and either pLTR-HIV3' or pCMV-HIV3'.

15   Cells are transfected using 5 to 10 mg each of the
resistance test vector and the appropriate packaging
expression vector(s) to produce resistance test vector host
cells. 293/T7RNAP-NLS cells are transfected by the calcium
phosphate coprecipitation method and Jurkat/T7RNAP-NLS cells
20   are transfected by electroporation. Individual anti-viral
agents, or combinations thereof, are added to individual
plates of transfected cells at the time of their
transfection, at an appropriate range of concentrations.
Twenty-four to 72 hours after transfection, cells are
25   collected by centrifugation, washed three times with
ice-cold phosphate-buffered saline, and assayed for firefly
luciferase activity as described. As transfected cells in
the culture do not efficiently express the indicator gene,
transfected cells in the culture, as well superinfected
30   cells in the culture, can serve as target host cells for
indicator gene expression. The reduction in luciferase
activity observed for cells transfected in the presence of
a given anti-viral agent, or agents as compared to a control
run in the absence of the anti-viral agent(s), generally
35

relates to the log of the concentration of the anti-viral
agent as a sigmoidal curve. This inhibition curve is used
to calculate the apparent inhibitory concentration (IC) of
that agent, or combination of agents, for the viral target
5   product encoded by the patient-derived segments present in
the resistance test vector.

EXAMPLE 2

HIV Drug Susceptibility And Resistance Tests
10 Using Resistance Test Vectors Comprising
Patient-derived   Segments   And   A
Non-Functional   Indicator   Gene   With   A
Permuted Coding Region

15 *Indicator Gene Viral Vector - Construction*
The genomic indicator gene viral vectors with patient
sequence   acceptor   sites,   pLG-lucPC-HS,   pLG-lucPC-PB,
pCG-lucPC-HS and pCG-lucPC-PB, and resistance test vectors
20 derived therefrom, each contain the following elements in a
5' to 3' orientation (Fig 4B): 1) an HIV-LTR U3 region
(pLG-lucPC-HS and pLG-lucPC-PB) or a first CMV IE
enhancer-promoter (pCG-lucPC-HS and pCG-lucPC-PB), 2) the
HIV-LTR R and U5 regions, 3) the coding regions of the HIV
25 *gag-pol, vif, vpr, tat, rev, vpu,* deleted *env,* and *nef*
genes, 4) a first indicator gene cassette containing the 5'
coding region of the luciferase gene, inserted into the
deleted *env* gene, 5) a second indicator gene cassette
containing the 3' coding region of the luciferase gene,
30 inserted into a deleted 3' HIV-LTR U3 region, and 6) a 3'
HIV-LTR R and U5 region. pLG-lucPC-HS and pCG-lucPC-HS
contain unique HpaI and SalI patient sequence acceptor sites
at nucleotides 2243 and 4190 of HXB2, respectively;
pLG-lucPC-PB and pCG-lucPC-PB contain unique PvuI and BamHI
35 patient sequence acceptor sites at nucleotides 2221 and 4212
of HXB2, respectively (see Example 1 for details). The
first indicator gene cassette contains: 1) a second CMV

enhancer-promoter, 2) the 5' coding region of the luciferase gene (amino acids 1 to 446), and 3) a CMV IE splice donor. The second indicator gene cassette contains: 1) an _-globin gene second exon splice acceptor, 2) the 3' coding region of the luciferase gene (amino acids 447 to 550), and 3) an SV40 polyadenylation site. The transcriptional orientation of the luciferase 5' and 3' coding regions are identical to one another, and opposite to that of the first CMV enhancer-promoter and viral LTRs. However, as the luciferase 5' and 3' coding regions are permuted in the resistance test vectors (ie., the 5' coding region is downstream of the 3' coding region), no mRNA is transcribed which can be spliced to generate a functional luciferase coding region. Following reverse transcription and strand transfer, the luciferase 3' coding region is copied from the 3' LTR to the 5' LTR, permitting the transcription of mRNA which can be spliced to generate a functional luciferase coding region (Fig 4C).

The subgenomic indicator gene viral vectors with patient sequence acceptor sites, pLS-lucPC-HS, pLS-lucPC-PB, pCS-lucPC-HS and pCS-lucPC-PB, and resistance test vectors derived therefrom, each contain the following elements in a 5' to 3' orientation (Fig 4B): 1) an HIV-LTR U3 region (pLS-lucPC-HS and pLS-lucPC-PB) or a first CMV IE enhancer-promoter (pCS-lucPC-HS and pCS-lucPC-PB), 2) the HIV-LTR R and U5 regions, 3) the coding region of the HIV gag-pol gene, 4) a first indicator gene cassette containing the 5' coding region of the luciferase gene, 5) an RRE element from the HIV env gene containing a viral packaging sequence, 6) a second indicator gene cassette containing the 3' coding region of the luciferase gene, inserted into a deleted 3' HIV-LTR U3 region, and 7) a 3' HIV-LTR R and U5 region. pLS-lucPC-HS and pCS-lucPC-HS contain unique HpaI and SalI patient sequence acceptor sites at nucleotides 2243

and 4190 of HXB2, respectively; pLS-lucPC-PB and pCS-lucPC-PB contain unique PvuI and BamHI patient sequence acceptor sites at nucleotides 2221 and 4212 of HXB2, respectively. The first indicator gene cassette contains:

5   1) a second CMV enhancer-promoter, 2) the 5' coding region of the luciferase gene (amino acids 1 to 446), and 3) a CMV IE splice donor. The second indicator gene cassette contains: 1) an (-globin gene second exon splice acceptor, 2) the 3' coding region of the luciferase gene (amino acids

10   447 to 550), and 3) an SV40 polyadenylation site. As the luciferase 5' and 3' coding regions are permuted in the resistance test vectors, reverse transcription and strand transfer must occur to generate non-permuted luciferase 5' and 3' coding regions, permitting the transcription of mRNA

15   which can be spliced to generate a functional luciferase coding region (Fig 4C).

     Plasmid pVL-luc5', which contains the first indicator gene cassette, is prepared in three steps. In the first two

20   steps, the artificial intron contained in pVL-1 consisting of the CMV IE splice donor and (-globin gene splice acceptor is subjected to site-directed mutagenesis to create restriction sites which upon digestion yield a DNA fragment whose 5' and 3' termini correspond precisely to the start

25   and end of the artificial intron. In step one, site-directed mutagenesis is carried out with pVL-1 using oligonucleotide 45 which contains the following sequences (5' to 3'): 1) the 18 nucleotide sequence which precedes the CMV IE splice donor, 2) a TAC trinucleotide sequence

30   corresponding to first half of the SnaBI restriction site, and 3) the 18 nucleotide sequence at the beginning of the artificial intron. As the sequence of the first three nucleotides of the intron is GTA, the resulting plasmid pVL-SnaBI contains a SnaBI restriction site which upon

35   digestion releases the 5' sequence of the intron as a blunt

DNA end. In step two, site-directed mutagenesis is carried out with pVL-SnaBI using oligonucleotide 46 which contains the following sequences (5' to 3'): 1) the 18 nucleotide sequence at the end of the artificial intron, 2) a CTG trinucleotide sequence corresponding to last half of the PvuII restriction site, and 3) the 18 nucleotide sequence following the _-globin splice acceptor. As the sequence of the last three nucleotides of the intron is CAG, the resulting plasmid pVL-SnaBI/PvuII contains a PvuII restriction site which upon digestion releases the 3' sequence of the intron as a blunt DNA end. In the third step, plasmid pVL-luc5' is prepared from two DNA fragments: 1) a vector DNA of 5.3 kB prepared by digesting plasmid pVL-luc with EcoRV and NheI, and treating the resulting vector with Klenow DNA polymerase and alkaline phosphatase, and 2) a DNA fragment of 0.1 kB containing the CMV IE splice donor, prepared by digesting plasmid pVL-SnaBI/PvuII with SnaBI and SmaI. Clones corresponding to pVL-luc5', which contain the CMV IE splice donor inserted in the correct orientation into the luciferase coding region, are identified by restriction mapping.

Plasmid pVL-luc3', which contains the second indicator gene cassette, is prepared in three steps. In step one, plasmid pBS-LTR, in which the 3'LTR of pBS-HXB2 is subcloned, is prepared from two DNA fragments: 1) a vector DNA of 3.0 kB prepared by digesting plasmid pBluescript II KS (+) with XhoI and XbaI, and 2) a DNA fragment of 0.8 kB containing the 3'LTR, prepared by digesting pBS-HXB2 with XhoI and XbaI. In step two, plasmid pBS-LTR-luc3', which contains the 3' coding region of luciferase followed by an SV40 polyadenylation site, inserted into the deleted 3'LTR, is prepared from two fragments: 1) a vector DNA of 3.5 kB prepared by digesting pBS-LTR with EcoRV, and treating the resulting vector with alkaline phosphatase, and 2) a DNA

fragment of 0.5 kB containing the 3' luciferase coding
region and SV40 polyA site, prepared by digesting plasmid
pVL-luc with EcoRV and NheI, followed by treatment of the
resulting fragment with Klenow DNA polymerase. Clones
'5   having the 3' luciferase coding region inserted in the
correct orientation (ie., opposite to the direct of
transcription in the 3'LTR) are identified by restriction
mapping. In step three, plasmid pVL-luc3' is prepared from
two DNA fragments: 1) a vector DNA of 4.0 kB prepared by
10   digesting plasmid pBS-LTR-luc3' with EcoRV, followed by
treatment of the resulting vector with alkaline phosphatase,
and 2) a DNA fragment of 0.1 kB containing the (-globin gene
second exon splice acceptor, prepared by digesting plasmid
pVL-SnaBI/PvuII with PvuII and SmaI. Clones corresponding
15   to pVL-luc3', which contain the (-globin second exon splice
acceptor inserted in the correct orientation into the
luciferase coding region, are identified by restriction
mapping.

20   Plasmids pLG-lucPC-HS, pLG-lucPC-PB, pLS-lucPC-HS and
pLS-lucPC-PB are prepared by the same three step procedure.
In step one, plasmids pLG-lucDP-HS, pLG-lucDP-PB,
pLS-lucDP-HS and pLS-lucDP-PB are prepared from two DNA
fragments: 1) a vector DNA prepared by digesting plasmids
25   pLG-lucPP-HS, pLG-lucPP-PB, pLS-lucPP-HS, pLS-lucPP-PB,
respectively, with SmaI and ClaI, and 2) a DNA fragment of
0.8 kB prepared by digesting plasmid pLG with SmaI and ClaI.
In step two, plasmids pLG-luc5'-HS, pLG-luc5'-PB,
pLS-luc5'-HS and pLS-luc5'-PB are prepared from two DNA
30   fragments: 1) a vector DNA prepared by digesting plasmids
pLG-lucDP-HS, pLG-lucDP-PB, pLS-lucDP-HS and pLS-lucDP-PB,
respectively, and treating the resulting vectors with
alkaline phosphatase, and 2) a DNA fragment of 2.5 kB
containing the first indicator gene cassette prepared by
35   digesting pVL-luc5' with NotI. Clones which contain the

first indicator gene cassette inserted into the viral vector
with a transcriptional orientation opposite to that of the
viral LTRs are identified by restriction mapping. In step
three, plasmids pLG-lucPC-HS, pLG-lucPC-PB, pLS-lucPC-HS and
pLS-lucPC-PB are prepared from two DNA fragments: 1) a
vector DNA prepared by digesting plasmids pLG-luc5'-HS,
pLG-luc5'-PB, pLS-luc5'-HS and pLS-luc5'-PB, respectively,
with XhoI and XbaI, and 2) a DNA fragment of 1.1 kB
containing the second indicator gene cassette, prepared by
digesting plasmid pVL-luc3' with XhoI and XbaI.

Plasmids pCG-lucPC-HS, pCG-lucPC-PB, pCS-lucPC-HS and
pCS-lucPC-PB are each prepared from two DNA fragments: 1) a
vector DNA prepared by digesting either plasmids
pLG-lucPC-HS, pLG-lucPC-PB, pLS-lucPC-HS and pLS-lucPC-PB,
respectively, with SmaI and ClaI, and 2) a DNA fragment of
1.3 kB prepared by digesting plasmid pCG with SmaI and ClaI.

Resistance Test Vector - Construction

Resistance test vectors containing a non-functional
indicator gene with a permuted coding region were designed
using the HIV genomic and subgenomic viral vectors
comprising anti-viral target genes described in Example 1.

Resistance test vectors are prepared from plasmids
pLG-lucPC-HS, pLG-lucPC-PB, pCG-lucPC-HS, pCG-lucPC-PB,
pLS-lucPC-HS, pLSlucPC-PB, pCS-lucPC-HS and pCS-lucPC-PB
(Fig. 4B), by the procedure described in Example 1.
Resistance test vectors are constructed with vectors
prepared from plasmids pLG-lucPC-HS, pCG-lucPC-HS,
pLS-lucPC-HS or pCS-lucPC-HS using amplified patient
sequences prepared with oligonucleotides 18 and 19, and with
oligonucleotides 22 and 23. Resistance test vectors are
constructed with vectors prepared from plasmids
pLG-lucPC-PB, pCG-lucPC-PB, pLS-lucPC-PB or pCS-lucPC-PB

-113-

using amplified patient sequences prepared with oligonucleotides 20 and 21, and with oligonucleotides 24 and 25.

5   *Drug Susceptibility and Resistance Test*
       Resistance tests are carried out by the procedures described
       in Example 1 as follows. Resistance test vectors prepared
       from plasmids pLG-lucPC-HS, pLG-lucPC-PB, pCG-lucPC-HS and
       pCG-lucPC-PB lack a functional HIV *env* gene, and are used in
10   conjunction with the packaging expression vector
       pVL-env4070A. Resistance test vectors prepared from
       plasmids pLS-lucPC-HS, pLS-lucPC-PB, pCS-lucPC-HS and
       pCS-lucPC-PB encode the HIV *gag-pol* gene products only, and
       are used in conjunction with pVL-env4070A, and either the
15   pLTR-HIV3' or pCMV-HIV3' packaging expression vectors. In
       resistance tests carried out using two host cell types, the
       293 cell line, the tsa54 cell line, the tsa201 cell line, or
       the BOSC 23 cell line are employed as packaging host cells,
       and unmodified Jurkat cells are employed as target cells.
20   As the non-functional indicator genes with permuted coding
       regions contained within these resistance test vectors are
       not efficiently expressed upon transfection of the packaging
       host cells, infection of target host cells is carried out
       either by co-cultivation with packaging host cells, or by
25   using virus from the packaging host cell supernatant. For
       similar reasons, resistance tests carried out with these
       resistance test vectors may employ a single host cell type.
       Resistance tests using a single host cell type are carried
       out using either 293, tsa54, tsa201, BOSC 23 or Jurkat
30   cells.

EXAMPLE 3

**HIV Drug Susceptibility And Resistance Test
Using Resistance Test Vectors Comprising
Patient-derived   Segment(s)   And   A
Non-Functional   Indicator   Gene   With   An
Inverted Intron**

Resistance test vectors containing a non-functional
indicator gene with an inverted intron were designed using
the HIV genomic and subgenomic viral vectors comprising
anti-viral target genes described in Example 1.

Indicator Gene Viral Vectors - Inverted Intron
The genomic indicator gene viral vectors with patient
sequence acceptor sites, pLG-lucII-HS, pLG-lucII-PB,
pCG-lucII-HS and pCG-lucII-PB, and resistance test vectors
derived therefrom, each contain the following elements in a
5' to 3' orientation (Fig 5B): 1) an HIV-LTR U3 region
(pLG-lucII-HS and pLG-lucII-PB) or a first CMV IE
enhancer-promoter (pCG-lucII-HS and pCG-lucII-PB), 2) the
HIV-LTR R and U5 regions, 3) the coding regions of the HIV
gag-pol, *vif*, *vpr*, *tat*, *rev*, *vpu*, deleted *env*, and *nef*
genes, 4) an indicator gene cassette inserted into the
deleted *env* gene, and 5) a 3' HIV-LTR. pLG-lucII-HS and
pCG-lucII-HS contain unique HpaI and SalI patient sequence
acceptor sites at nucleotides 2243 and 4190 of HXB2,
respectively; pLG-lucII-PB and pCG-lucII-PB contain unique
PvuI and BamHI patient sequence acceptor sites at
nucleotides 2221 and 4212 of HXB2, respectively (see Example
1 for details). The indicator gene cassette contains 1) a
second CMV enhancer-promoter, 2) the coding region of the
luciferase gene interrupted by an inverted artificial
intron, and 3) an SV40 polyadenylation sequence. The
overall transcriptional orientation of the indicator gene
cassette is opposite to that of the first CMV
enhancer-promoter and viral LTRs, while the orientation of
the artificial intron is the same as the latter elements.

-115-

Transcription of the indicator gene by the second CMV enhancer-promoter does not lead to the production of functional transcripts as the inverted intron cannot be spliced in this orientation. Transcription of the indicator gene by the 5' viral LTR or the first CMV IE enhancer-promoter, however, leads to the removal of the inverted intron by RNA splicing, although the indicator gene is still not functionally expressed as the resulting transcript has an antisense orientation. Following the reverse transcription of this transcript and integration of the resultant proviral DNA, the indicator gene can be functionally transcribed by the second CMV enhancer-promoter as the inverted intron has been previously removed (Fig 5C).

The subgenomic indicator gene viral vectors with patient sequence acceptor sites, pLS-lucII-HS, pLS-lucII-PB, pCS-lucII-HS and pCS-lucII-PB, and resistance test vectors derived therefrom, each contain the following elements in a 5' to 3' orientation (Fig 5B): 1) an HIV-LTR U3 region (pLS-lucII-HS and pLS-lucII-PB) or a first CMV IE enhancer-promoter (pCS-lucII-HS and pCS-lucII-PB), 2) the HIV-LTR R and U5 regions, 3) the coding region of the HIV gag-pol gene, 4) the indicator gene cassette, 5) an RRE element from the HIV env gene containing a viral packaging sequence, and 6) a 3' HIV-LTR. pLS-lucII-HS and pCS-lucII-HS contain unique HpaI and SalI patient sequence acceptor sites at nucleotides 2243 and 4190 of HXB2, respectively; pLS-lucII-PB and pCS-lucII-PB contain unique PvuI and BamHI patient sequence acceptor sites at nucleotides 2221 and 4212 of HXB2, respectively. The indicator gene cassette contains 1) a second CMV enhancer-promoter, 2) the coding region of the luciferase gene interrupted by an inverted artificial intron, and 3) an SV40 polyadenylation sequence. As is the case for the pLG-lucII and pCG-lucII genomic viral vectors, the indicator

-116-

genes of pLS-lucII and pCS-lucII cannot be functionally transcribed until after the inverted intron is removed and reverse transcription and proviral integration occur (Fig 5C).

Plasmid pVL-lucII, which contains the indicator gene cassette, in which the artificial intron from pVL-SnaBI/PvuII is inserted into the luciferase coding region in an inverted orientation, is prepared from two DNA fragments: 1) a vector fragment of 5.8 kB prepared by digesting pVL-luc with EcoRV and treating the resulting vector with alkaline phosphatase, and 2) a DNA fragment of 0.2 kB corresponding precisely to the artificial intron sequence prepared by digesting pVL-SnaBI/PvuII with SnaI and PvuII. Clones corresponding to pVL-lucII which contain the artificial intron inserted into the luciferase coding region in an inverted orientation are identified by restriction mapping.

Plasmids pLG-lucII-HS, pLG-lucII-PB, pLS-lucII-HS and pLS-lucII-PB are prepared from two DNA fragments: 1) a vector DNA prepared by digesting plasmids pLG-lucDP-HS, pLG-lucDP-PB, pLS-lucDP-HS and pLS-lucDP-PB, respectively, and treating the resulting vectors with alkaline phosphatase, and 2) a DNA fragment of 3.2 kB containing the luciferase indicator gene cassette prepared by digesting pVL-lucII with NotI. Clones which contain the correct plucII indicator gene cassette inserted into the viral vector with a transcriptional orientation opposite to that of the viral LTRs are identified by restriction mapping and are used for the preparation of resistance test vectors.

Plasmids pCG-lucII-HS, pCG-lucII-PB, pCS-lucII-HS and pCS-lucII-PB are each prepared from two DNA fragments: 1) a vector DNA prepared by digesting plasmids pLG-lucII-HS,

pLG-lucII-PB, pLS-lucII-HS and pLS-lucII-PB, respectively,
with SmaI and ClaI, and 2) a DNA fragment of 1.3 kB prepared
by digesting plasmid pCG with SmaI and ClaI.

Resistance Test Vector - Construction

Resistance test vectors are prepared from plasmids
pLG-lucII-HS, pLG-lucII-PB, pCG-lucII-HS, pCG-lucII-PB,
pLS-lucII-HS, pLS-lucII-PB, pCS-lucII-HS and pCS-lucII-PB
(Fig. 5B), by the procedure described in Example 1.
Resistance test vectors are constructed with vectors
prepared from plasmids pLG-lucII-HS, pCG-lucII-HS,
pLS-lucII-HS or pCS-lucII-HS using amplified patient
sequences prepared with oligonucleotides 18 and 19, and with
oligonucleotides 22 and 23. Resistance test vectors are
constructed with vectors prepared from plasmids
pLG-lucII-PB, pCG-lucII-PB, pLS-lucII-PB or pCS-lucII-PB
using amplified patient sequences prepared with
oligonucleotides 20 and 21, and with oligonucleotides 24 and
25.

Drug Susceptibility and Resistance Test

Resistance tests are carried out by the procedures described
in Example 1 as follows. Resistance test vectors prepared
from plasmids pLG-lucII-HS, pLG-lucII-PB, pCG-lucII-HS and
pCG-lucII-PB lack a functional HIV *env* gene, and are used in
conjunction with the packaging expression vector
pVL-env4070A. Resistance test vectors prepared from
plasmids pLS-lucII-HS, pLS-lucII-PB, pCS-lucII-HS and
pCS-lucII-PB encode the HIV *gag-pol* gene products only, and
are used in conjunction with pVL-env4070A, and either the
pLTR-HIV3' or pCMV-HIV3' packaging expression vectors. In
resistance tests carried out using two host cell types, the
293 cell line, the tsa54 cell line, the tsa201 cell line, or
the BOSC 23 cell line are employed as packaging host cells,
and unmodified Jurkat cells are employed as target cells.

-118-

As the non-functional indicator genes with inverted introns contained within these resistance test vectors are not efficiently expressed upon transfection of the packaging host cells, infection of target host cells is carried out either by co-cultivation with packaging host cells, or by using virus from the packaging host cell supernatant. For similar reasons, resistance tests carried out with these resistance test vectors may employ a single host cell type. Resistance tests using a single host cell type are carried out using either 293, tsa54, tsa201, BOSC 23 or Jurkat cells.

EXAMPLE 4

Non-Particle Based HIV Drug Susceptibility And Resistance Test Using Resistance Test Vectors Comprising Patient-derived Segment(s) And A Non-functional Indicator Gene.

Drug Susceptibility and Resistance Test

Non-particle based resistance tests are carried out using the resistance test vectors comprising non-functional indicator genes with either permuted promoters, permuted coding regions or inverted introns, described in Examples 1, 2 and 3. These non-particle based resistance tests are performed by transfection of a single host cell type with each resistance test vector in the absence of packaging expression vectors. Although the non-functional indicator genes contained within these resistance test vectors are not efficiently expressed upon transfection of the host cells, there is detectable indicator gene expression resulting from non-viral particle-based reverse transcription. Reverse transcription and strand transfer results in the conversion of the permuted, non-functional indicator gene to a non-permuted, functional indicator gene. As reverse transcription is completely dependent upon the expression of the *pol* gene contained within each resistance test vector,

anti-viral agents may be tested for their ability to inhibit the *pol* gene products encoded by the patient-derived segments contained within the resistance test vectors. Non-particle based resistance tests are carried out by the general procedures described in Example 1 with the following modifications: 1) Resistance test vectors are transfected into the appropriate host cells, 2) anti-viral agents, or combinations thereof, are added at appropriate concentrations to individual cultures of transfected host cells immediately after transfection, 3) host cells are harvested 24 to 72 hours following transfection and assayed for luciferase activity. The reduction in luciferase activity observed for host cells transfected with a given resistance test vector in the presence of a given anti-viral agent, or agents, as compared to a control run in the absence of the anti-viral agent(s) is used to calculate the apparent inhibitory content (Ki) of that agent, or combination of agents, for the viral target gene product encoded by the patient-derived segments present in the resistance test vector.

Resistance Test Vector - Construction

For non-particle based resistance tests with resistance test vectors comprising a non-functional indicator gene with a permuted promoter, resistance test vectors are prepared as described in Example 1 using plasmids pLG-lucPP-HS, pLG-lucPP-PB, pCG-lucPP-HS, pCG-lucPP-PB, pLS-lucPP-HS, pLS-lucPP-PB, pCS-lucPP-HS or pCS-lucPP-PB. Each resistance test vector is transfected into host cells expressing a cytoplasmic T7 RNA polymerase (eg., 293/T7RNAP cells or Jurkat/T7RNAP cells). Such host cells are prepared by the stable transfection of 293 cells and Jurkat cells as described in Example 1, using plasmid pVL-T7RNAP, which directs the expression of a cytoplasmic phage T7 RNA polymerase in human and other mammalian cells and cell

-120-

lines. pVL-T7RNAP is constructed from the following two DNA fragments: 1) a vector fragment of 4.3 kB prepared by digestion of the mammalian expression vector pVL-2 with EcoRI and BglII, and 2) a DNA fragment of 2.6 kB containing

5 the complete coding region of the T7 RNA polymerase (nucleotides 166 to 2815, coordinates given in GenBank accession number M38308, Grachev and Pletnev (1984) *Bioorg. Khim.* 10, 824-843) prepared by PCR using plasmid pT7-G1 as a template with oligonucleotides 47 and 40 as primers,

10 followed by digestion with EcoRI and BglII. Oligonucleotide 47 incorporates a unique EcoRI site followed by a consensus sequence for eukaryotic translation initiation (e.g., Kozak (1991) *J. Biol. Chem,* **266,** 19867-19870), while oligonucleotide 40 incorporates a unique BglII site.

15

For non-particle based resistance tests with resistance test vectors comprising a non-functional indicator gene with a permuted coding region or inverted intron, resistance test vectors are prepared as described in Example 1 using

20 plasmids pLG-lucPC-HS, pLG-lucPC-PB, pCG-lucPC-HS, pCG-lucPC-PB, pLS-lucPC-HS, pLS-lucPC-PB, pCS-lucPC-HS, pCS-lucPC-PB, pLG-lucII-HS, pLG-lucII-PB, pCG-lucII-HS, pCG-lucII-PB, pLS-lucII-HS, pLS-lucII-PB, pCS-lucII-HS or pCS-lucII-PB. Each resistance test vector is transfected

25 into either 293, tsa54, tsa201, BOSC 23 or Jurkat cells.

EXAMPLE 5

HIV Drug Susceptibility And Resistance Test Using Resistance Test Vectors Comprising
30 **Patient-derived Segment(s) And A Functional Indicator Gene.**

Indicator Gene Viral Vector - Functional Indicator Gene
The genomic indicator gene viral vectors with patient
35 sequence acceptor sites, plasmids pLG-luc-HS-1, pLG-luc-HS-2, pLG-luc-PB-1, pLG-luc-PB-2, pCG-luc-HS-1,

-121-

pCG-luc-HS-2, pCG-luc-PB-1 and pCG-luc-PB-2, and resistance test vectors derived therefrom, each contain the following elements in a 5' to 3' orientation (Fig 6): 1) an HIV-LTR U3 region (pLG-luc-HS-1, pLG-luc-HS-2, pLG-luc-PB-1 and pLG-luc-PB-2) or a first CMV IE enhancer-promoter (pCG-luc-HS-1, pCG-luc-HS-2, pCG-luc-PB-1 and pCG-luc-PB-2), 2) the HIV-LTR R and U5 regions, 3) the coding regions of the HIV gag-pol, vif, vpr, tat, rev, vpu, deleted env, and nef genes, 4) an indicator gene cassette inserted into the deleted env gene, and 5) a 3' HIV-LTR. pLG-luc-HS-1, pLG-luc-HS-2, pCG-luc-HS-1 and pCG-luc-HS-2 contain unique HpaI and SalI patient sequence acceptor sites at nucleotides 2243 and 4190 of HXB2, respectively; pLG-luc-PB-1, pLG-luc-PB-2, pCG-luc-PB-1 and pCG-luc-PB-2 contain unique PvuI and BamHI patient sequence acceptor sites at nucleotides 2221 and 4212 of HXB2, respectively (see Example 1 for details). The indicator gene cassettes of each plasmid contain 1) a second CMV enhancer-promoter, 2) the coding region of the luciferase gene, and 3) an SV40 polyadenylation sequence. The indicator gene cassettes of pLG-luc-HS-1, pLG-luc-PB-1, pCG-luc-HS-1 and pCG-luc-PB-1 are inserted into the vector with a transcriptional orientation opposite to the viral LTRs or first CMV enhancer-promoter (Fig. 6B), while the indicator gene cassettes of pLG-luc-HS-2, pLG-luc-PB-2, pCG-luc-HS-2 and pCG-luc-PB-2 are inserted into the vector with the same orientation (Fig. 6C).

The subgenomic indicator gene viral vectors with patient sequence acceptor sites, plasmids pLS-luc-HS-1, pLS-luc-HS-2, pLS-luc-PB-1, pLS-luc-PB-2, pCS-luc-HS-1, pCS-luc-HS-2, pCS-luc-PB-1 and pCS-luc-PB-2, and resistance test vectors derived therefrom, each contain the following elements in a 5' to 3' orientation (Fig 6): 1) an HIV-LTR U3 region (pLS-luc-HS-1, pLS-luc-HS-2, pLS-luc-PB-1 and

-122-

pLS-luc-PB-2) or a first CMV IE enhancer-promoter (pCS-luc-HS-1, pCS-luc-HS-2, pCS-luc-PB-1 and pCS-luc-PB-2), 2) the HIV-LTR R and U5 regions, 3) the coding region of the HIV *gag-pol* gene, 4) the indicator gene cassette, 5) an RRE
5 element from the HIV *env* gene containing a viral packaging sequence, and 6) a 3' HIV-LTR. pLS-luc-HS-1, pLS-luc-HS-2, pCS-luc-HS-1 and pCS-luc-HS-2 contain unique HpaI and SalI patient sequence acceptor sites at nucleotides 2243 and 4190 of HXB2, respectively; pLS-luc-PB-1, pLS-luc-PB-2,
10 pCS-luc-PB-1 and pCS-luc-PB-2 contain unique PvuI and BamHI patient sequence acceptor sites at nucleotides 2221 and 4212 of HXB2, respectively. The indicator gene cassettes of each plasmid contain 1) a second CMV enhancer-promoter, 2) the complete coding region of the luciferase gene, and 3) an
15 SV40 polyadenylation sequence. The indicator gene cassettes of pLS-luc-HS-1, pLS-luc-PB-1, pCS-luc-HS-1 and pCS-luc-PB-1 are inserted into the vector with a transcriptional orientation opposite to the viral LTRs or first CMV enhancer-promoter (Fig. 6B), while the indicator gene
20 cassettes of pLS-luc-HS-2, pLS-luc-PB-2, pCS-luc-HS-2 and pCS-luc-PB-2 are inserted into the vector with the same orientation (Fig. 6C).

Plasmids pLG-luc-HS-1 and pLG-luc-HS-2, pLG-luc-PB-1 and
25 pLG-luc-PB-2, pCG-luc-HS-1 and pCG-luc-HS-2, pCG-luc-PB-1 and pCG-luc-PB-2, pLS-luc-HS-1 and pLS-luc-HS-2, pLS-luc-PB-1 and pLS-luc-PB-2, pCS-luc-HS-1 and pCS-luc-HS-2, pCS-luc-PB-1 and pCS-luc-PB-2 are each prepared from two DNA fragments: 1) a vector DNA prepared by
30 digesting either pLG-lucII-HS, pLG-luII-PB, pCG-lucII-HS, pCG-lucII-PB, pLS-luc-HS, pLS-lucII-PB, pCS-lucII-HS and pCS-lucII-PB, respectively with NotI and treating the resulting vectors with alkaline phosphatase, and 2) a DNA fragment of 3.0 kB containing the luciferase indicator gene
35 cassette prepared by digesting pVL-luc with NotI. Clones

-123-

containing the indicator gene cassette inserted into a given
viral vector in both transcriptional orientations relative
to the viral LTRs are identified by restriction mapping
(eg., pLG-luc-HS-1 and pLG-luc-HS-2).

5

Resistance Test Vector - Construction
Resistance test vectors containing a functional indicator
gene were designed using the HIV genomic and subgenomic
viral vectors comprising anti-viral target genes described
10   in Example 1. Resistance test vectors are prepared from the
above plasmids (Fig. 6) by the procedure described in
Example 1. Resistance test vectors are constructed with
vectors prepared from plasmids pLG-luc-HS-1, pLG-luc-HS-2,
pCG-luc-HS-1, pCG-luc-HS-2, pLS-luc-HS-1, pLS-luc-HS-2,
15   pCS-luc-HS-1 or pCS-luc-HS-1 using amplified patient
sequences prepared with oligonucleotides 18 and 19, and with
oligonucleotides 22 and 23. Resistance test vectors are
constructed with vectors prepared from plasmids
pLG-luc-PB-1, pLG-luc-PB-2, pCG-luc-PB-1, pCG-luc-PB-2,
20   pLS-luc-PB-1, pLS-luc-PB-2, pCS-luc-PB-1 or pCS-luc-PB-1
using amplified patient sequences prepared with
oligonucleotides 20 and 21, and with oligonucleotides 24 and
25.

25   *Drug Susceptibility and Resistance Test*
Resistance tests are carried out by the procedures described
in Example 1 as follows. Resistance test vectors prepared
from plasmids pLG-luc-HS-1, pLG-luc-HS-2, pLG-luc-PB-1,
pLG-luc-PB-2, pCG-luc-HS-1, pCG-luc-HS-2, pCG-luc-PB-1 and
30   pCG-luc-PB-2 lack a functional HIV *env* gene, and are used in
conjunction with the packaging expression vector
pVL-env4070A. Resistance test vectors prepared from
plasmids pLS-luc-HS-1, pLS-luc-HS-2, pLS-luc-PB-1,
pLS-luc-PB-2, pCS-luc-HS-1, pCS-luc-HS-2, pCS-luc-PB-1 and
35   pCS-luc-PB-2 encode the HIV *gag-pol* gene products only, and

-124-

are used in conjunction with pVL-env4070A, and either the
pLTR-HIV3' or pCMV-HIV3' packaging expression vectors.
Resistance tests are carried out with two host cell types,
using the 293 cell line, the tsa54 cell line, the tsa201
5   cell line, or the BOSC 23 cell line as packaging host cells,
and using unmodified Jurkat cells as target cells.   The
infection of target cells with these or other resistance
test vectors containing functional indicator genes is
carried out employing the procedure for infection with
10   resistance test vector viral particles from the filtered
supernatants obtained from resistance test vector indicator
host cells, as described in Example 1.   In contrast to
resistance test vectors comprising non-functional indicator
gene viral vectors with a permuted promoter or an inverted
15   intron, those comprising a functional indicator genes are
typically able to express their indicator genes in the
transfected packaging host cells.   Neither the
co-cultivation procedure, nor the resistance test using a
single cell type can therefore be readily adapted for the
20   infection of target cells using resistance test vectors with
functional indicator genes, as it would be difficult to
distinguish between indicator gene expression in infected
target host cells and the transfected packaging host cells.

25   All publications and patent applications cited in this
specification are herein incorporated by reference in their
entirety as if each individual publication or patent
application were specifically and individually indicated to
be incorporated by reference.

30

As will be apparent to those skilled in the art to which the
invention pertains, the present invention may be embodied in
forms other than those specifically disclosed above, for
example to carry out the drug susceptibility and resistance
35   test on other viruses, without departing from the spirit or

essential characteristics of the invention. The particular
embodiments of the invention described above, are, therefore
to be considered as illustrative and not restrictive. The
scope of the present invention is as set forth in the
5   appended claims rather than being limited to the examples
contained in the foregoing description.

EXAMPLE 6

HIV Drug Susceptibility And Resistance
10   Test Using Resistance Test Vectors
Comprising Patient-derived Segment(s)
And A Functional Indicator Gene.

Drug Susceptibility and Resistance Tests

15   Resistance tests were carried out with resistance test
vectors based on the indicator gene viral vectors
pCG-CXCN(F-lucP)2; and pCG-CXAT(F-lucP)2, both of which are
similar to the pCG-luc-2 described in Example 5, using two
host cell types. In the case of pCG-CXCN(F-lucP)2 the
20   indicator gene viral vector was modified in that the
indicator gene cassette lacked intron A (CMV/α-globin intron
described above), not contain a polyadenylation signal and
the downstream 3' U5 sequence was ommitted in the
construction. The downstream U5 was replaced with the SV40
25   poly A signal and origin of replication regions. The
indicator gene viral vector pCG-CXAT(F-lucP)2 differed from
pCG-CXCN(F-lucP)2 in that the indicator gene cassette
contained an artificial intron downstream of the CMV
enhancer-promoter and a TK polyadenylation signal region.
30   Resistance test vector viral particles were produced by a
first host cell (the resistance test vector host cell) that
was prepared by transfecting a packaging host cell with the
resistance test vector and the packaging expression vector.
The resistance test vector viral particles were then used to
35   infect a second host cell (the target host cell) in which
the expression of the indicator gene is measured.

AZT Drug Susceptibility/Resistance Tests

The resistance test vector pCG-luc-2 containing a functional luciferase gene cassette was constructed and host cells were transfected with the resistance test vector DNA. The resistant test vectors contained "test" patient-derived reverse transcriptase sequences that were either susceptible or resistant to the nucleoside reverse transcriptase inhibitor, AZT (Sigma). The resistance test vector viral particles produced by transfecting the resistance test vector DNA into host cells were used to infect target host cells grown either in the absence of AZT or in the presence of increasing concentrations of the drug (ranging from approximately $0.0001\mu M$ to $1000\mu M$). The amount of luciferase activity produced in infected target host cells in the presence of drug was compared to the amount of luciferase produced in infected target host cells in the absence of drug. Drug resistance was measured as the amount of drug required to inhibit by 50% the luciferase activity detected in the absence of drug (inhibitory concentration 50%, IC_{50}). The IC_{50} values were determined by plotting percent drug inhibition vs. $\log_{10}$ drug concentration.

Host cells (293) were seeded in 10-cm-diameter dishes and were transfected several days after plating with resistance test vector plasmid DNA and the envelope expression vector pCXAS(4070A-env). Transfections were performed using a calcium-phosphate precipitation procedure. The cell culture media containing the DNA precipitate was replaced with fresh medium, from one to 24 hours, after transfection. Cell culture media containing resistance test vector viral particles was harvested one to four days after transfection and was passed through a 0.45-mm filter before being stored at -80° C. HIV capsid protein (p24) levels in the harvested cell culture media were determined by an EIA method as

-127-

described by the manufacturer (SIAC; Frederick, MD). Six to forty-eight hours before infection, target cells (293 and 293/T) were plated in cell culture media containing no AZT or serial two fold dilutions of AZT beginning at 100μM and ending at 0.00005μM. The AZT concentrations were maintained throughout the infection. Target host cells were inoculated with 90μl of transfected resistance test vector host cell supernatant. Control infections were performed using cell culture media from mock transfections (no DNA) or transfections containing the resistance test vector plasmid DNA without the envelope expression plasmid DNA (pCXAS(4070A-env)). One to twenty-four hours after the inoculation, fresh medium was added to each well. Twelve to thirty-six hours later the media was completely replaced with fresh media. One to three or more days after infection the media was removed and cell lysis buffer (Promega) was added to each well. Cell lysates were diluted 100 fold in lysis buffer and each diluted cell lysate was assayed for luciferase activity (Fig. 7A). The inhibitory effect of AZT was determined using the following equation:

$$\text{\% luciferase inhibition} = 1 - (RLUluc_{[AZT]} \div RLUluc)$$

where $RLUluc_{[AZT]}$ is the Relative Light Unit of luciferase activity in infected cells in the presence of AZT and RLUluc is the Relative Light Unit of luciferase activity in infected cells in the absence of AZT. IC_{50} values were obtained from the sigmoidal curves that were generated from the data by plotting the percent inhibition of luciferase activity vs. the log_{10} drug concentration. The AZT inhibition curves are shown in (Fig. 7B).

Nevirapine Drug Susceptibility/Resistance Test
The resistant test vector, based on the indicator gene viral

vector pCG-CXCN(F-lucP)2, contained the reverse transcriptase sequence derived from the biologically active proviral clone, pNL4-3, that is susceptible to the non-nucleoside reverse transcriptase inhibitor, nevirapine (BI-RG-587, Boehringer Ingleheim). Transfection of host packaging cells and infection of host target cells were performed as described for the AZT drug susceptibility/resistance tests as described above. Nevirapine susceptibility/resistance was evaluated using nevirapine concentrations ranging from 0.0001μM to 100μM. The nevirapine inhibition curve was determined as described above for AZT and is shown in Figure 7C.

Indinavir Drug Susceptibility/Resistance Tests

The resistant test vector, based on the indicator gene viral vector pCG-CXCN(F-lucP)2, contained the protease sequence derived from the biologically active proviral clone, pNL4-3, that is susceptible to the protease inhibitor, indinavir (MK-639, Merck). Transfection of host packaging cells and infection of host target cells were performed as described for the AZT drug susceptibility/resistance test except that the protease inhibitor, indinavir, was present in the transfected packaging host cell cultures as well as the infected target host cell cultures, as described above. Indinavir susceptibility/resistance was evaluated using indinavir concentrations ranging from 1.5pM to 3μM. The indinavir inhibition curve was determined as described above for AZT and is shown in Figure 7D.

EXAMPLE 7

**Hepatitis Drug Susceptibility And Resistance
Test Using Resistance Test Vectors
Comprising Patient-Derived Segment(s) And A
Non- Functional Indicator Gene Containing An
Inverted Intron.**

Indicator Gene Viral Vector - Construction

Indicator gene viral vectors containing a non-functional
indicator gene with an inverted intron were designed using
HBV subgenomic viral vectors comprising viral genes which
are the target(s) of anti-viral drugs. The indicator gene
viral vectors pCS-HBV(NF-IG)II-(PSAS-), are based on the
subgenomic viral vector pCS-HBV. The indicator gene viral
vector contains a non-functional indicator gene cassette
containing an inverted intron and all of the cis-acting
regulatory elements that are necessary for HBV DNA
replication (i.e. (DR1, 5'ε DR2, DR1*, 3'pA) but lacks the
HBV gene sequences (i.e. C, P, S, X genes) that provide the
trans-acting structural and enzymatic functions that are
necessary for HBV DNA replication and virus particle
formation (Fig. 8B). The C, P, S and X genes and patient
sequence acceptor sites are contained within a packaging
vector pPK-CPX (described below, Fig. 8D) and pPK-S
(described below, Fig. 8E). In this embodiment the
indicator gene viral vector pCS-HBV(NF-IG)II-(PSAS-) and the
packaging vector pPK-CPX constitute a resistance test vector
system. The non-functional indicator gene viral vector
pCS-HBV(NF-IG)II-(PSAS-) contains the following elements in
a 5' to 3' orientation: (1) the CMV IE enhancer promoter
region, (2) the 5' region of the HBV genome and the DR1 and
5' copy of the encapsidation signal region (ε) (the pre-C
ORF translation initiation codon is eliminated), (3) a
non-functional indicator gene cassette in which the
indicator gene ORF contains an inverted intron, (4) the
region of the HBV genome containing DR2, DR1*, the 3'ε, and

the 3' HBV polyadenylation (pA) signal region. The non-functional indicator gene expression cassette is comprised of some or all of the following elements arranged in the 5' to 3' orientation: (1) a transcriptional enhancer-promoter region, (2) an intron, (3) an indicator gene containing an inverted intron, (4) a transcriptional polyadenylation signal sequence (e.g. SV40), (HSV-1 thymidine kinase gene). The indicator gene expression cassette has a transcriptional orientation opposite to the HBV sequence elements (Fig. 8B). However, the intron within the indicator gene ORF has the same transcriptional orientation as the HBV sequence elements.

In a second embodiment, the non-functional indicator gene viral vector, pCS-HBV(NF-IG)II(PSAS+), contains a non-functional indicator gene cassette containing an inverted intron, all of the cis-acting regulatory elements that are necessary for HBV DNA replication, and some or all of the HBV gene sequences (i.e. C, P, S, X genes) that provide the trans-acting structural and enzymatic functions that are necessary for HBV DNA replication and virus particle formation (Fig. 8F). Resistance test vectors derived from the indicator gene viral vector pCS-HBV(NF-IG)II(PSAS+) contain patient sequence acceptor sites (PSAS) and are used in conjunction with the packaging vector, pPK-CSX (Fig. 8H). In this embodiment the indicator gene viral vector may also provide some or all of the packaging functions, such as P. The structural and enzymatic activities that are not provided by the indicator gene viral vector, but that are necessary for HBV DNA replication and virus particle formation, are provided using additional packaging vector(s) pPK-CSX (described below, Fig. 8H). In this embodiment, the non-functional indicator gene viral vector, pCS-HBV(NF-IG)II(PSAS+) contains the following elements in a 5' to 3' orientation: (1) the CMV IE

-131-

enhancer-promoter region, (2) the 5' region of the HBV
genome and the DR1 and 5'ε (the pre-C ORF translation
initiation codon is eliminated), (3) an indicator gene
cassette, containing an inverted intron, positioned within
the region of the HBV genome which may contain some or all
of the C, P, S, and X genes as well as a patient-derived P
gene segment, (4) the region of the HBV genome containing
DR2, DR1*, the 3'ε, and the 3'ε HBV pA signal region.
Within the non-functional indicator gene viral vector,
pCS-HBV(NF-IG)II-(PSAS+) the indicator gene expression
cassette has a transcriptional orientation opposite to the
HBV sequence elements (Fig. 8F). However, the intron within
the indicator gene ORF has the same transcriptional
orientation as the HBV sequence elements.

In transfected cells the packaging vectors (Figs 8D, 8E and
8H) provide, in trans, the structural and enzymatic
functions that are necessary for HBV DNA replication and
virus particle formation, but that are not provided by the
resistance test vector or the indicator gene viral vector.
In the embodiment in which an indicator gene viral vector
such as pCS-HBV(NF-IG)PP-(PSAS-) is co-transfected with a
packaging vector, such as pPK-CPX, the combination of those
vectors constitute a resistance test vector system, it is
the packaging vector that contains patient sequence acceptor
sites for insertion of the patient-derived P gene segment
(described above). The packaging vector pPK-CPX contains
the following elements in a 5' to 3' orientation: (1) the
CMV IE enhancer-promoter region, (2) the region of the HBV
genome spanning from the C ORF translation initiation codon
to the 3' pA signal and including the C, P, S, and X genes.
The C gene of the packaging vectors is modified such that it
does not contain and/or express the pre-C ORF sequences and
does not express the S proteins (described below).

-132-

In HBV, RNA that encodes C and/or P proteins is
preferentially packaged, in cis, and consequently could
interfere with the efficient packaging of the resistance
test vector containing a non-functional indicator gene or a
non-functional indicator gene viral vector RNA that does not
encode C and P proteins. Two steps are taken to prevent the
encapsidation of RNA produced from packaging vectors and to
improve the encapsidation efficiency of the resistance test
vector or non-functional indicator gene viral vector RNA.
First, the RNA produced by packaging vectors does not
contain the 5' encapsidation signal region (ε) (Figs 8D, 8E
and 8H). Second, in cases where either the C gene and/or P
gene packaging functions are provided by the resistance test
vector or non-functional indicator gene viral vector,
packaging vectors, such as pPK-S or pPK-CSK, that do not
express the C and/or P gene products are used (Figs. 8E and
8H).

Resistance Test Vectors - Construction

Resistance test vectors are prepared by 1) modifying the
indicator gene viral vector pCS-HBV(NF-IG)II-(PSAS-) by
introducing unique restriction sites, called patient
sequence acceptor sites (PSAS) in the P gene region, 2)
amplifying patient-derived segments corresponding to the HBV
drug target, e.g. reverse transcriptase or DNA polymerase,
by the amplification of viral DNA present in the serum or
cells of infected patients, and 3) inserting the amplified
sequences precisely into the indicator gene viral vectors at
patient sequence acceptor sites (Fig. 8F). Alternatively,
resistance test vector systems are prepared by 1) modifying
the packaging vectors, pPK-CPX, by introducing patient
sequence acceptor sites in the P gene, 2) amplifying
patient-derived segments corresponding to the HBV drug
target, e.g. reverse transcriptase or DNA polymerase, by
amplification using viral DNA present in the serum or cells

-133-

of infected patients, and 3) inserting the amplified
sequences precisely into the packaging vectors at patient
sequence acceptor sites (Fig. 8D). In one embodiment, the
5' PSAS is situated near the border of the spacer and RT
5   domains of the P protein (immediately downstream of the S
protein translation initiation site) and the 3' PSAS is
situated near the C-terminal end of the RNase H domain of
the P protein. Insertion of a patient-derived P gene
segment into the patient sequence acceptor sites results in
10   the formation of a chimeric P gene sequence in which the TP.
and spacer domains are encoded by the vector P gene sequence
while the reverse transcriptase/polymerase and RNase H
domains are encoded by the patient-derived segments (Figs 8D
and 8F).

15

In HBV the entire S gene ORF overlaps the P gene ORF but is
expressed using a different reading frame (Nassal, M. and
Schaller, H. (1993) *Trends in Microbiology* 1, 221-228).
Thus, HBV P gene sequences (reverse transcriptase and RNase
20   H domains) obtained from patients also contain the
corresponding patient S gene sequences. Expression of the
patient-derived S gene region from the overlapping S gene
ORF is prevented by eliminating the three S gene ORF
(pre-S1, pre-S2, and S) translation initiation sites and/or
25   introducing in-frame termination codons in the
pCS-HBV(NF-IG)II-(PSAS+) or pPK-CPK vectors (Figs. 8F and
8D). S gene expression is provided, in trans, using a
separate packaging vector that provides well-characterized
S gene products (Figs 8E and 8H). The modifications that
30   eliminate S gene expression are performed without
introducing changes to the overlapping amino acid sequences
that are encoded by the terminal protein (TP) or spacer
domains of the P gene.

35

-134-

Drug Susceptibility and Resistance Tests

Drug susceptibility and resistance tests are carried out
with a resistance test vector based on indicator gene viral
vectors pCS-HBV(NF-IG)II-(PSAS+) or with a resistance test
vector system based on an indicator gene viral vector,
pCS-HBV(NF-IG)II-(PSAS-) and a packaging vector, pPK-CPX,
using either one type of host cell or two types of host
cell. Co-transfection of packaging host cells either with
a resistance test vector, such as pCS-HBV(NF-IG)II-(PSAS+)
and a packaging vector, pPK-CSX, or with an indicator gene
viral vector, such as pCS-HBV(NF-IG)II-(PSAS-) and packaging
vector-containing patient-derived segment, such as pPK-CPX,
(i.e. resistance test vector system) produces HBV viral
particles containing an encapsidated indicator gene
"pregenome" RNA, which as a result of splicing of the
inverted intron, contains a functional indicator gene.
(Figs. 8B and 8C).

Replicate transfections are performed on a series of
packaging host cell cultures maintained either in the
absence of the anti-viral drug or in increasing
concentrations of the anti-HBV drug (e.g., an HBV P protein
reverse transcriptase or polymerase inhibitor). After
maintaining the packaging host cells for up to several days
in the presence or absence of the anti- HBV drug the level
of drug susceptibility or resistance can be assessed either
directly in the host packaging cell lysates, or in isolated
HBV particles obtained by harvesting the host packaging cell
culture media. Alternative approaches can be used to
evaluate drug susceptibility and resistance in the cell
lysates and the isolated HBV particles.

In one embodiment, referred to as the one cell assay, drug
susceptibility or resistance is assessed by measuring
indicator gene expression, e.g. luciferase activity, in the

transfected packaging host cells in the presence or absence
of anti-viral drug.   A reduction in luciferase activity
observed for cells transfected in the presence of a given
anti-viral agent, or combination of agents as compared to a
5   control run in the absence of the anti-viral agent(s),
generally relates to the log of the concentration of the
anti-viral agent as a sigmoidal curve.

In a second embodiment, referred to as the two cell assay,
10.   drug susceptibility or resistance is assessed by measuring
indicator gene expression, e.g. luciferase activity, in the
target host cells following infection or transfection with
HBV particles or HBV particle DNA, respectively.   HBV viral
particles obtained from packaging host cells are used to
15   infect the target host cells or DNA from those particles is
used to transfect target host cells.   At the time of
infection or transfection the appropriate concentration of
the anti-viral drug is added to the cell culture.   Up to
several days following the infection or transfection, the
20   target host cells are lysed and indicator gene expression is
measured.   A reduction in indicator gene expression will be
observed for cells transfected or infected in the presence
of drugs which inhibit HBV replication, for example by
inhibiting either the reverse transcriptase (- strand DNA)
25   or the DNA polymerase (+ strand DNA) activities of the HBV
P protein as compared to a control run in the absence of
drug.

In a third embodiment, referred to as the DNA structure
30   indicator assay, drug susceptibility or resistance is
assessed by measuring the level of HBV DNA replication that
has occurred within the transfected packaging host cells or
within the virus particles produced by the packaging host
cells.   In transfected host cells the HBV subgenomic viral
35   vector is transcribed and the RNA transcript is packaged as

-136-

"pregenomic" RNA. During virus maturation, the pregenomic
RNA is converted to the relaxed circular form of the genomic
DNA (rc-DNA) consisting of a complete minus strand and
partial plus strand DNA copy. In a subsequent step, the
5   rc-DNA is converted to a covalently closed circular DNA form
(cccDNA). To measure HBV DNA replication, amplification
primers are designed to amplify an HBV DNA structure that is
formed by the splicing of pregenomic RNA and the conversion
of this spliced RNA to the rc-DNA and cccDNA forms.

10

Formation of the correct amplification target structure
within HBV particles requires successful completion of HBV
DNA replication resulting in the formation of rc-DNA and
cccDNA. Anti-viral drugs that inhibit HBV DNA replication
15   (reverse transcription and DNA polymerase activities) will
limit the formation of the DNA target sequence, which in
turn, can be measured as a decrease in the amplified DNA
product using a number of quantitative amplification assays.
In one example (Fig. 10B), the binding site of the reverse
20   primer (Pr) is separated into two components by an intron
sequence that is inserted into an indicator gene ORF in the
same transcriptional orientation as the HBV sequences. The
primer binding site of the forward primer (Pf) is located
within the region of the viral vector that is flanked by the
25   DR2 and DR1* sequences. In the linear HBV vector the Pf and
Pr primers direct DNA synthesis in opposite directions and
are oriented outward with respect to each other. In this
case, the Pr primer directs DNA synthesis in the upstream
direction (toward the 5' copy of (ε) and the Pf primer
30   directs DNA synthesis in the downstream direction (toward
the 3' (ε). This arrangement of primers and template does
not constitute a functional amplification unit in the linear
indicator gene viral vector. Furthermore, the Pr binding
site is not intact in the linear unspliced vector and thus
35   Pr will not anneal to the target DNA. In contrast, the Pf

-137-

and Pr primers assume an inward orientation with respect to each other in the relaxed circle form of HBV DNA found in mature virions and splicing of the RNA pregenome has assembled an intact Pr primer binding site. Both primers now direct DNA synthesis toward the single copy of DR1 within the plus strand copy of rc-DNA and in either plus or minus strand or cccDNA. This arrangement of primers and template constitutes a functional amplification unit (Fig. 10C).

In an alternative embodiment of the DNA sequence indicator assay (Fig. 9D) the 5' exonuclease activity of the amplification enzyme (e.g. Taq polymerase) is measured rather than the production of amplified DNA (C. Heid et al., 1996. *Genome Research* 6:986-994). The 5' exonuclease activity is measured by monitoring the nucleolytic cleavage of a fluorescently tagged oligonucleotide probe that is capable of binding to the amplified DNA template region flanked by the Pf and Pr binding sites. The performance of this assay is dependent on the close proximity of the 3' end of the upstream primer (Pf) to the 5' end of the oligonucleotide probe. When the Pf primer is extended it displaces the 5' end of the oligonucleotide probe such that the 5' exonuclease activity of the polymerase cleaves the oligonucleotide probe. The purpose of the intron is to distance the Pf binding site sufficiently far away from the 5' end of the exonuclease probe sequence to essentially eliminate detectable exonuclease digestion of the probe oligonucleotide in the unspliced target template. Removing the intron by splicing serves to position the 3' end of the Pf binding site immediately upstream of the probe 5' binding site. The latter rearrangement enables the quantitative detection of exonuclease activity of the amplified target template (Fig. 1E).

Drug Screening

Drug screening is carried out using an indicator gene viral
vector containing a non-functional indicator gene cassette
with an inverted intron and a packaging vector(s).   In
5   transfected packaging host cells, the indicator gene viral
vector produces an encapsidation competent (ε+) RNA
transcript containing the indicator gene.   The packaging
vector(s) provide, in trans, the structural and/or enzymatic
viral functions that are not provided by the resistance test
10   vector, but that are necessary for viral DNA replication and
particle formation. Upon co-transfection of packaging host
cells, the indicator gene viral vector and packaging vectors
give rise to HBV viral particles containing an encapsidated
indicator gene viral vector "pregenome" RNA, which as a
15   result of splicing of the inverted intron, contains a
functional indicator gene.

Drug screening is performed as follows: indicator gene viral
vector and packaging vector DNA is used to transfect the
20   packaging host cells. Replicate transfections are performed
on a series of packaging host cell cultures maintained
either in the absence or presence of potential anti-viral
compounds   (e.g.,   candidate HBV P protein reverse
transcriptase or polymerase inhibitors). After maintaining
25   the packaging host cells for up to several days in the
presence or absence of the candidate anti-viral drugs the
level of inhibition of DNA replication is assessed either
directly in the packaging host cell lysates, or in isolated
HBV particles obtained by harvesting the host packaging cell
30   culture media.   Either DNA detection or indicator gene
activity methods, described above, can be used to evaluate
potential anti-HBV drug candidates.

EXAMPLE 8

Hepatitis Drug Susceptibility And Resistance Test Using Resistance Test Vectors Comprising Patient-Derived Segment(s) And A Non-Functional Indicator Gene Containing A Permuted Promoter.

Indicator Gene Viral Vector - Construction

Indicator gene viral vectors containing a non-functional indicator gene with a permuted promoter were designed using an HBV subgenomic viral vector comprising viral genes which are the target(s) of anti-viral drugs. The indicator gene viral vectors, pCS-HBV(NF-IG)PP-(PSAS-), are based on the subgenomic viral vector pCS-HBV. The indicator gene viral vector, pCS-HBV(NF-IG)PP-(PSAS-), contains a non-functional indicator gene cassette with a permuted promoter and all of the cis-acting regulatory elements that are necessary for HBV DNA replication (i.e. DR1, 5'ε DR2, DR1*, 3'pA) but lacks the HBV gene sequences (i.e. C, P, S, X genes) that provide the trans-acting structural and enzymatic functions that are necessary for HBV DNA replication and virus particle formation (Fig. 10B). The C, P and X and patient sequence acceptor sites are contained within a packaging vector pPK-CPX (described in Example 7, see Fig. 8D). The S gene is contained within a packaging vector pPK-S (described in Example 7, see Fig. 8E). In this embodiment, the indicator gene viral vector, pCS-HBV(NF-IG)PP-(PSAS-) and the packaging vector pPK-CPX constitute a resistance test vector system. The non-functional indicator gene viral vector, pCS-HBV(NF-IG)PP-(PSAS-), contains the following elements in a 5' to 3' orientation: (1) the CMV IE enhancer promoter region, (2) the 5' region of the HBV genome and the DR1 and 5'ε (the pre-C ORF translation initiation codon is eliminated), (3) a non-functional indicator gene cassette assembled such that the promoter region is positioned 3',

-140-

i.e. downstream, of the indicator gene ORF, (4) the 3'
region of the HBV genome containing DR2, DR1*, the 3'ε, and
the 3' HBV pA signal region (the pre-C ORF translation
initiation codon is eliminated). The non-functional
5   indicator gene expression cassette is comprised of some or
all of the following elements arranged in the 5' to 3'
orientation: (1) an internal ribosome entry site (IRES),
(2) an indicator gene, which may contain an inverted intron,
and (3) a transcriptional polyadenylation signal sequence
10   (e.g. HSV-1 thymidine kinase gene, SV40) (4) an
enhancer-promoter region. Within the non-functional
indicator gene viral vector, the indicator gene expression
cassette has a transcriptional orientation either opposite
to or the same as the HBV sequence elements. In cases where
15   the indicator gene ORF contains an intron, the intron has
the same transcriptional orientation as the HBV sequence
elements.

In a second embodiment, the non-functional indicator gene
20   viral vector contains a non-functional indicator gene
cassette containing a permuted promoter region, all of the
cis-acting regulatory elements that are necessary for HBV
DNA replication, and some or all of the HBV gene sequences
(i.e. C, P, S, X genes) that provide the trans-acting
25   structural and enzymatic functions that are necessary for
HBV DNA replication and virus particle formation,
pCS-HBV(NF-IG)PP-(PSAS+) (Fig. 10D). Resistance test vectors
derived from the indicator gene viral vector,
pCS-HBV(NF-IG)PP-(PSAS+), contain patient sequence acceptor
30   sites and are used in conjunction with the packaging vector
pPK-CSK (Fig. 8H). In this embodiment the indicator gene
viral vector may also provide some or all of the packaging
functions. Furthermore, in this embodiment the structural
and enzymatic activities that are not provided by the
35   indicator gene viral vector, but that are necessary for HBV

-141-

DNA replication and virus particle formation, are provided
using additional packaging vectors. In this embodiment, the
non-functional   indicator   gene   viral   vector,
pCS-HBV(NF-IG)PP-(PSAS+), contains the following elements in
a 5' to 3' orientation: (1) the CMV IE enhancer-promoter
region, (2) the 5' region of the HBV genome and the DR1 and
5'ε (the pre-C ORF translation initiation codon is
eliminated), (3) an enhancer-promoter region (permuted
promoter), (4) the P gene containing patient-derived segment
(5) the indicator gene ORF (6) an internal ribosome entry
site (IRES), and (7) the 3' region of the HBV genome
containing DR2, DR1*, the 3'ε, and the 3' HBV pA signal
region (the pre-C ORF translation initiation codon is
eliminated). Within the non-functional indicator gene viral
vector, the indicator gene expression cassette has a
transcriptional orientation either in the reverse or forward
direction with respect to the HBV sequence elements. In
cases where the indicator gene contains an inverted intron,
the intron has a transcriptional orientation the same as to
the HBV sequence elements.

Resistance Test Vector - Construction
Resistance test vectors containing a non-functional
indicator gene with a permuted promoter were designed using
the HBV subgenomic viral vector comprising anti-viral target
genes as described in Example 8. The indicator gene viral
vector or the packaging vector is modified to include
patient sequence acceptor sites (PSAS) for the insertion of
P gene containing patient-derived segments (PDS) (described
in Example 7, see Figs. 8D and 8F). The expression of the
patient-derived S gene is eliminated as described in Example
7 (see Fig. 8D). Uniform S gene expression is provided, in
trans, using a separate packaging vector that provides
well-characterized S gene products (Fig. 8E).

-142-

Drug Susceptibility and Resistance Test

Drug susceptibility and resistance tests are carried out with a resistance test vector based on indicator gene viral vectors, pCS-HBV(NF-IG)PP-(PSAS+), or with a resistance test

5   vector system comprising an indicator gene viral vector, pCS-HBV(NF-IG)PP-(PSAS-) and a packaging vector, pPK-CPX, using either one type of host cell or two types of host cell. Upon co-transfection of packaging host cells, either with a resistance test vector and a packaging vector or with

10   an indicator gene viral vector and packaging vectors (i.e. resistance test vector system) HBV viral particles are produced containing an encapsidated indicator gene "pregenome" RNA containing a non-functional indicator gene. Within the transfected host cells, the non-functional

15   indicator gene with the permuted promoter is converted to a functional indicator gene during the HBV DNA replication process (Figs. 10B and 10C).

Drug susceptibility and resistance tests are performed as

20   described in Example 7 (above). The resistance or susceptibility of patient-derived reverse transcriptase and/or DNA polymerase activities to various anti-viral drugs can be measured by measuring the levels of indicator gene expression in transfected or infected host cells.

25   Alternatively, resistance or susceptibility can be measured by quantitating the amount of HBV DNA replication that has taken place. The latter can be performed using quantitative DNA amplification assays. In one example of this type of assay, (Figs. 9F and 9G) the primer binding site of the

30   reverse primer (Pr) is located in the region downstream of the 5'ε. The primer binding site of the forward primer (Pf) is located within the region flanked by the DR2 and DR1* sequences. In the linear HBV vector the Pf and Pr primers direct DNA synthesis in opposite directions. In this case,

35   the Pr primer directs DNA synthesis in the upstream

direction (toward the 5'ε and the Pf primer directs DNA
synthesis in the downstream direction (toward the 3'ε. This
primer configuration does not constitute a functional
amplification unit in the linear copy of the viral vector
5   that is used for transfection. In contrast, the Pf and Pr
primers assume an orientation toward each other in the
rc-DNA form that is found in mature virions. Both primers
now direct DNA synthesis toward the single copy of DR1
within the plus strand copy of rc-DNA. This arrangement of
10   primers and template constitutes a functional amplification
unit.

Drug Screening
Drug screening using an indicator gene viral vector that
15   contains a non-functional indicator gene with a permuted
promoter is performed essentially as described in Example 7
above.

EXAMPLE 9
20   **Hepatitis Drug Susceptibility And Resistance
Test Using Resistance Test Vectors
Comprising Patient-derived Segments And A
Non-Functional Indicator Gene Containing A
Permuted Promoter And Translation Initiation
25   Sites.**

Indicator Gene Viral Vector
Indicator gene viral vectors containing a non-functional
30   indicator gene with a permuted promoter and translation
initiation site, pCS-HBV(NF-IG)PPTIS were designed using HBV
subgenomic viral vector comprising viral genes which are the
target(s) of anti-viral drugs. The indicator gene viral
vectors pCS-HBV(NF-IG)PPTIS(PSAS-), are based on the
35   subgenomic viral vector pCS-HBV. The indicator gene viral
vector contains a non-functional indicator gene cassette
with permuted promoter and translation initiation regions,

-144-

and all of the cis-acting regulatory elements that are necessary for HBV DNA replication (i.e. DR1, 5'ε, DR2, DR1*, 3'pA) but lacks the HBV gene sequences (i.e. C, P, S, X genes) that provide the trans-acting structural and

5   enzymatic functions that are necessary for HBV DNA replication and virus particle formation (Fig. 11B). The C, P and X genes and patient sequence acceptor sites are contained within a packaging vector pPK-CPX (Example 7, Fig. 8D). The S gene is contained in the packaging vector,

10   pPK-S, (Example 7, Fig. 8E). In this embodiment the indicator gene viral vector pCS-HBV(NF-IG)PPTIS(PSAS-) and the packaging vector pPK-CPX constitute a resistance test vector system. The non-functional indicator gene viral vector, pCS-HBV(NF-IG)PPTIS(PSAS-), contains the following

15   elements in a 5' to 3' orientation: (1) the CMV IE enhancer-promoter region, (2) the 5' region of the HBV genome including the DR1 and 5'ε (the pre-C ORF translation initiation codon is eliminated), (3) an indicator gene ORF lacking a translation initiation site, (4) an

20   enhancer-promoter region (permuted promoter) (5) the 3' region of the HBV genome containing DR2, a functional pre-C ORF translation initiation codon, DR1*, the 3'ε, and the 3' HBV pA signal region. The non-functional indicator gene cassette is comprised of some or all of the following

25   elements arranged in a 5' to 3' orientation: (1) an indicator gene ORF that does not contain an in-frame translation initiation site, (2) a transcriptional polyadenylation signal sequence (e.g. HSV-1 thymidine kinase gene, SV40), (3) an enhancer-promoter region. Within the

30   non-functional indicator gene viral vector, pCS-HBV(NF-IG)PPTIS (PSAS-) the indicator gene expression cassette transcriptional orientation is the same as the HBV sequence elements. In cases where the indicator gene ORF contains an intron, the intron has a transcriptional

35   orientation the same as the HBV sequence elements.

In a second embodiment, the non-functional indicator gene
viral vector contains a non-functional indicator gene
cassette containing a permuted promoter and translation
initiation regions, all of the cis-acting regulatory
5   elements that are necessary for HBV DNA replication, and
some or all of the HBV gene sequences (i.e. C, P, S, X
genes) that provide the trans-acting structural and
enzymatic functions that are necessary for HBV DNA
replication and virus particle formation,
10   pCS-HBV(NF-IG)PPTIS(PSAS+) (Fig. 11D). The structural and
enzymatic activities that are not provided by the indicator
gene viral vector, but that are necessary for HBV DNA
replication and virus particle formation, are provided using
additional packaging vectors pPK-CSX (described in Example
15   7, see Fig. 8E). The C, S and X are contained within the
packaging vector pPK-CSX (described in Example 7, see Fig.
8H). In this embodiment, the non-functional indicator gene
viral vector, pCS-HBV(NF-IG)PPTIS(PSAS+), contains the
following elements in a 5' to 3' orientation (Fig. 11D): (1)
20   the CMV IE enhancer-promoter region, (2) the 5' region of
the HBV genome including the DR1 and 5'ε (the pre-C ORF
translation initiation codon is eliminated), (3) an
indicator gene ORF lacking a translation initiation site,
(4) the P gene containing patient-derived segment, (5) an
25   enhancer-promoter region (permuted promoter) (6) the 3'
region of the HBV genome containing DR2, pre-C ORF
translation initiation codon, DR1*, the 3'ε, and the 3' HBV
pA signal region. Within the non-functional indicator gene
viral vector, the indicator gene expression cassette has a
30   transcriptional orientation the same as the HBV sequence
elements. In cases where the indicator gene contains an
inverted intron, the intron has a transcriptional
orientation the same as the HBV sequence elements.

35

-146-

Resistance Test Vectors - Construction

Resistance test vectors containing a non-functional indicator gene with a permuted promoter were designed using the HBV subgenomic viral vector comprising anti-viral target genes as described in Example 7. The indicator gene viral vector or the packaging vector is modified to include patient sequence acceptor sites (PSAS) for the insertion of P gene containing patient-derived segments (PDS) (described in Example 7, see Figs. 8D and 8F). The expression of the patient-derived S gene is eliminated as described in Example 7 (see Fig. 8D). Uniform S gene expression is provided, in trans, using a separate packaging vector that provides well-characterized S gene products (Fig. 8E).

Drug Susceptibility and Resistance Test

Drug susceptibility and resistance tests are carried out by the procedures described in Examples 7 and 8. The non-functional indicator gene is converted to a functional indicator gene during HBV replication (Figs. 11B and 11C).

Drug Screening

Drug screening using an indicator gene viral vector that contains a non-functional indicator gene with permuted promoter and translation initiation regions is performed essentially as described in Examples 7 and 8 (above).

EXAMPLE 10

Hepatitis Drug Susceptibility And Resistance Test Using Resistance Test Vectors Comprising Patient-derived Segments And A Non-Functional Indicator Gene With Permuted Coding Regions.

Indicator Gene Viral Vector- Indicator gene viral vectors containing a non-functional indicator gene with permuted coding region were designed using HBV subgenomic viral

vectors comprising viral genes which are the target(s) of anti-viral drugs. The indicator gene viral vectors pCS-HBV(NF-IG)PCR(PSAS-), are based on the subgenomic viral vector pCS-HBV. The indicator gene viral vector contains a non-functional indicator gene cassette with a permuted coding region, and all of the cis-acting regulatory elements that are necessary for HBV DNA replication (i.e. DR1, 5'ε DR2, DR1*, 3'pA) but lacks the HBV gene sequences (i.e. C, P, S, X genes) that provide the trans-acting structural and enzymatic functions that are necessary for HBV DNA replication and virus particle formation (Fig. 12B). The C, P and X genes and patient sequence acceptor sites are contained within a packaging vector, pPK-CPX and the S gene is provided by the packaging vector pPK-S (described in Example 7, see Figs. 8D and 8E). In this embodiment the indicator gene viral vector pCS-HBV(NF-IG)PCR(PSAS-) and the packaging vector pPK-CPX constitute a resistance test vector system. The non-functional indicator gene viral vector, pCS-HBV(NF-IG)PCR(PSAS-), contains the following elements in a 5' to 3' orientation: (1) the CMV IE enhancer-promoter region, (2) the 5' region of the HBV genome including the DR1 and 5'ε (the pre-C ORF translation initiation codon is eliminated), (3) a non-functional indicator gene cassette assembled such that the promoter region and a 5' portion of the coding region are positioned 3', i.e. downstream, of the remaining 3' portion of the coding region, (4) the 3' region of the HBV genome containing DR2, DR1*, the 3'ε, and the 3' HBV pA signal region (the pre-C ORF translation initiation codon is eliminated). The non-functional indicator gene cassette is comprised of some or all of the following elements arranged in the 5' to 3' orientation: (1) the 3' region of an intron ending in a splice acceptor sequence (2) the 3' region of an indicator (reporter) ORF or selectable marker ORF, (3) a transcriptional polyadenylation signal sequence (e.g. HSV-1 thymidine kinase gene, SV40), (4) an

enhancer-promoter region, (5) the 5' region of an indicator
gene ORF, (6) the 5' region of an intron beginning in a
splice donor sequence. Within the non-functional indicator
gene viral vector, the indicator gene expression cassette
5   has a transcriptional orientation either the same or
opposite the HBV sequence elements. In cases where the
indicator gene ORF contains an intron, the intron is
oriented in the same orientation with respect to the HBV
sequence elements.
10

In a second embodiment, the non-functional indicator gene
viral vector contains a non-functional indicator gene
cassette containing a permuted coding region, all of the
cis-acting regulatory elements that are necessary for HBV
15   DNA replication, and some or all of the HBV gene sequences
(i.e. C, P, S, X genes) that provide the trans-acting
structural and enzymatic functions that are necessary for
HBV DNA replication and virus particle formation
pCS-HBV(NF-IG)PCR(PSAS+) (Fig. 12D). Resistance test
20   vectors derived from the indicator gene viral vector
pCS-HBV(NF-IG)PCR(PSAS+) contain patient sequence acceptor
sites (PSAS) and are used in conjunction with the packaging
vector pPK-CSK (described in Example 7, see Fig. 8H). In
this embodiment the indicator gene viral vector may also
25   provide some or all of the packaging functions. In this
embodiment the structural and enzymatic activities that are
not provided by the indicator gene viral vector, but that
are necessary for HBV DNA replication and virus particle
formation, are provided using additional packaging vectors.
30   In this embodiment, the non-functional indicator gene viral
vector contains the following elements in a 5' to 3'
orientation: (1) the CMV IE enhancer promoter region, (2)
the region of the HBV genome immediately downstream of the
pre-C ORF translation initiation codon and the DR1 and 5'ε,
35   (3) the indicator gene cassette containing a region of the

HBV genome which may contain some or all of the C, P, S, and X genes, (4) the region of the HBV genome containing DR2, DR1*, the 3'ε, and the 3' HBV pA signal region (the pre-C ORF initiation codon has been eliminated). Within the

5   non-functional indicator gene viral vector, pCS-HBV(NF-IG)PCR(PSAS+) the indicator gene expression cassette has a transcriptional orientation either the same or opposite to HBV sequence elements. In cases where the indicator gene contains an inverted intron, the intron is

10 oriented in the same orientation with respect to the HBV sequence elements.

Resistance Test Vectors - Construction

Resistance test vectors containing a non-functional

15 indicator gene with a permuted coding region were designed using the HBV subgenomic viral vector comprising anti-viral target genes as described in Example 7. The indicator gene viral vector or the packaging vector is modified to include patient sequence acceptor sites (PSAS) for the insertion of

20 P gene containing patient-derived segments (PDS) (described in Example 7, see Figs. 8D and 8F). The expression of the patient-derived S gene is eliminated as described in Example 7 (see Fig. 8D). Uniform S gene expression is provided, in trans, using a separate packaging vector that provides

25 well-characterized S gene products (Fig. 8E).

Drug Susceptibility and Resistance Tests

Drug susceptibility and resistance tests are carried out by the procedures described in Examples 7 and 8. The

30 non-functional indicator gene is converted to a functional indicator gene during HBV replication (Figs. 12B and 12C).

Drug Screening

Drug screening using an indicator gene viral vector that

35 contains a non-functional indicator gene with permuted

promoter and translation initiation regions is performed
essentially as described in Examples 7 and 8 (above).

EXAMPLE 11

**Hepatitis Drug Susceptibility And Resistance
Test Using Resistance Test Vector Comprising
Patient-Derived Segment(s) And A Functional
Indicator Gene.**

Indicator Gene Viral Vector - Construction

Indicator gene viral vectors containing a functional
indicator gene were designed using HBV subgenomic viral
vectors comprising viral genes which are the target(s) of
anti-viral drugs. The indicator gene viral vectors
pCS-HBV(F-IG)(PSAS-), are based on the subgenomic viral
vector pCS-HBV. The indicator gene viral vector,
pCS-HBV(F-IG)(PSAS-), contains a functional indicator gene
cassette and all of the cis-acting regulatory elements that
are necessary for HBV DNA replication (i.e. DR1, 5'ε DR2,
DR1*, 3'pA) but lacks the HBV gene sequences (i.e. C, P, S,
X genes) that provide the trans-acting structural and
enzymatic functions that are necessary for HBV DNA
replication and virus particle formation (Fig. 14B). The C,
P and X genes and patient sequence acceptor sites are
contained within a packaging vector pPK-CPX and the S gene
is contained within the packaging vector, pPK-S, (described
below see Figs. 8D and 8E). In this embodiment the
indicator gene viral vector pCS-HBV(F-IG)(PSAS-) and the
packaging vector pPK-CPX constitute a resistance test vector
system. The functional indicator gene viral vector
pCS-HBV(F-IG)(PSAS-) contains the following elements in a 5'
to 3' orientation: (1) the CMV IE enhancer-promoter region,
(2) the 5' region of the HBV genome including the DR1 and
5'ε   (the pre-C ORF translation initiation codon is
eliminated), (3) a functional indicator gene cassette, (4)
the 3' region of the HBV genome containing DR2, DR1*, the
3'ε, and the 3' HBV pA signal region (the pre-C ORF

translation initiation codon is eliminated). The indicator gene expression cassette is comprised of some or all of the following elements arranged in the 5' to 3' orientation: (1) a transcriptional enhancer-promoter region, (2) an intron, (3) an indicator gene ORF or selectable marker gene ORF, (4) a transcriptional polyadenylation signal sequence (e.g. SV40) or unidirectional (HSV-1 thymidine kinase gene). The indicator gene expression cassette has a transcriptional orientation the same as or opposite to the HBV sequence elements.

In a second embodiment, the indicator gene viral vector contains a functional indicator gene cassette, all of the cis-acting regulatory elements that are necessary for HBV DNA replication, and some or all of the HBV gene sequences (i.e. C, P, S, X genes) that provide the trans-acting structural and enzymatic functions that are necessary for HBV DNA replication and virus particle formation pCS-HBV(F-IG)(PSAS+) (Fig. 13D). Resistance test vectors derived from the indicator gene viral vector pCS-HBV(F-IG)(PSAS+) contain patient sequence acceptor sites (PSAS) and are used in conjunction with the packaging vector pPK-CSK. In this embodiment the indicator gene viral vector also provides some or all of the packaging functions. The structural and enzymatic activities that are not provided by the indicator gene viral vector, but that are necessary for HBV DNA replication and virus particle formation, are provided using additional packaging vectors pPK-CSX (described in Example 7, see Fig. 8H). In this embodiment, the functional indicator gene viral vector contains the following elements in a 5' to 3' orientation: (1) the CMV IE enhancer promoter region, (2) the region of the HBV genome immediately downstream of the pre-C ORF translation initiation codon and the DR1 and 5'ε, (3) a functional indicator gene cassette within the region of the HBV genome

that contains some or all of the C, P, S, and X genes, (4)
the region of the HBV genome containing DR2, DR1*, the 3'ε,
and the 3' HBV pA signal region. Within the indicator gene
viral vector, the indicator gene expression cassette has a
5   transcriptional orientation either opposite to or the same
as the HBV sequence elements.

Resistance Test Vector - Construction
Resistance   test   vectors   containing   a   non-functional
10   indicator gene with a permuted promoter were designed using
the HBV subgenomic viral vector comprising anti-viral target
genes as described in Example 7. The indicator gene viral
vector or the packaging vector is modified to include
patient sequence acceptor sites (PSAS) for the insertion of
15   P gene containing patient-derived segments (PDS) (described
in Example 7, see Figs. 8D and 8F). The expression of the
patient-derived S gene is eliminated as described in Example
7 (see Fig. 8D). Uniform S gene expression is provided, in
trans, using a separate packaging vector that provides
20   well-characterized S gene products (Fig. 8E).

Drug Susceptibility and Resistance Test
Drug susceptibility and resistance test are carried out with
a resistance test vector based on a functional indicator
25   gene viral vector, pCS-HBV(F-IG)(PSAS+) or with a resistance
test vector system based on an indicator gene viral vector,
pCS-HBV(F-IG)(PSAS-) and a packaging vector(s). In
transfected packaging host cells, the indicator gene viral
vector produces an encapsidation competent (ε+) RNA
30   transcript containing a functional indicator gene cassette.
The packaging vector(s) provide, in trans, the structural
and/or enzymatic viral functions that are not provided by
the functional indicator gene viral vector, but that are
necessary for viral DNA replication and particle formation.
35   Upon co-transfection of packaging host cells, the indicator

gene viral vector and packaging vectors are capable of forming HBV particles containing an encapsidated indicator gene viral vector "pregenome" RNA containing a functional indicator gene cassette.

In this example, the indicator gene viral vector contains a functional indicator gene cassette and therefore can produce indicator gene activity in transfected cells in the absence of HBV DNA replication (Fig. 13). In the case of a functional indicator gene, the inhibition of HBV DNA replication by drugs can be evaluated by harvesting the virus particles produced in the packaging host cell and using the particles (or particle DNA) to infect (or transfect) a target host cell. Alternatively, DNA replication can be measured directly in virus particles isolated from packaging host cells by using the DNA as an indicator. A drug which inhibits HBV DNA replication will reduce the formation of virus particles containing the "mature" rc-DNA form of the functional indicator gene viral vector. Consequently, the functional indicator gene will not be efficiently transferred to the target host cells during infection/transfection and the amount of indicator gene viral vector cccDNA and indicator gene activity in these cells will be reduced. The detection of indicator gene expression in target host cells in a two cell assay is performed as described in Example 7. The detection of rc-DNA in virus particles is performed using DNA as an indicator as described in Example 8 and illustrated in Figs. 9F and 9G.

Drug Screening

Drug screening using an indicator gene viral vector that contains a non-functional indicator gene with permuted promoter and translation initiation regions is performed essentially as described in Examples 7 and 8 (above).

Oligonucleotides

1) 5'-AGTGAATTAGCCCTTCCACCCGGGTCGAGCTTGGCGTAATCA-3'
    (42-mer) (SEQ ID NO:1)

2) 5'-CTGTTGGGAAGGGCGATCTCTAGATGCTAGAGATTTTCCACA-3'
    (42-mer) (SEQ ID NO:2)

3) 5'-CTCCTCCTCCAAGTCTGAGCGGCCGCCTTTAGCATCTGATGCAC-3'
    (44-mer) (SEQ ID NO:3)

4) 5'-CTCCTCCTCCAAGTCTGAGCGGCCGCCATATGGTGTTTTACTAA-3'
    (44-mer) (SEQ ID NO:4)

5) 5'-GGTCTAACCAGAGAGACCCGGTTCACTAAACGAGCT-3'
    (36-mer) (SEQ ID NO:5)

6) 5'-GAATTCGCGGCCGCAATTCCGCCCCTCTCCCT-3'
    (32-mer)(SEQ ID NO:6)

7) 5'-GTTAACGCGGCCGCGATATAGTTCCTCCTTTC-3'
    (32-mer)(SEQ ID NO:7)

8) 5'-GAATTCTCGCGACCATGGAAGACGCCAAAAAC-3'
    (32-mer) (SEQ ID NO:8)

9) 5'-GTTAACAGATCTCTCGAGTTACAATTTGGACTTTCC-3'
    (36-mer) (SEQ ID NO:9)

10) 5'-AGACGGGCACACACTACTTAATACGACTCACTATAGGG
    TGAAGCACTCAAGGCAAG-3' (56-mer) (SEQ ID NO:10)

11) 5'-AAGAGTGACCTGAGGGAAGTTAACGGATACAGTTCCTTGTCT-3'
    (42-mer) (SEQ ID NO:11)

12) 5'-TCCAGCACTGACTAATTTGTCGACTTGTTCATTTCCTCCAAT-3'
    (42-mer) (SEQ ID NO:12)

13) 5'-TAACGCCTATTCTGCTATGCCGACACCCAATTCTGAAAATGG-3'
    (42-mer) (SEQ ID NO:13)

14) 5'-AAGGATACAGTTCCTTGTCGATCGGCTCCTGCTTCTGAGGGG-3'
    (42-mer) (SEQ ID NO:14)

15) 5'-CTAAAAATAGTACTTTCCGGATCCCAGCACTGACTAATTTAT-3'
    (42-mer) (SEQ ID NO:15)

16) 5'-TTAGCTCCTTCGGTCCTCCAATCGTTGTCAGAAGTAAGTTGG-3'
    (42-mer) (SEQ ID NO:16)

17) 5'-GTCCCAGATAAGTGCCAAGGATTCGTTCACTAATCGAATGGA-3'

(42-mer) (SEQ. ID NO:17)

18) 5'-GAATTCGTTAACTTCCCTCAGATCACTCTTTGG-3'

(33-mer) (SEQ ID NO:18)

19) 5'-GTTAACGTCGACTTGTTCATTTCCTCCAAT-3'

(30-mer) (SEQ ID NO:19)

20) 5'-GAATTCCGATCGACAAGGAACTGTATCCTTTAACTTCCC

TCAGATCACTCTTTGG-3' (55-mer) (SEQ ID NO:20)

21) 5'-GTTAACGGATCCCAGCACTGACTAATTTATCTACTTGTTC

ATTTCCTCCAAT-3' (52-mer) (SEQ ID NO:21)

22) 5'-GAATTCGTTAACTTCCCTCA(G/A)ATC(A/C)CTCTTTGG-3'

(33-mer pool) (SEQ ID NO:22)

23) 5'-GTTAACGTCGACTT(G/T)(T/C)TCATTTCCTCC(A/T)AT-3'

(30-mer pool) (SEQ ID NO:23)

24) 5'-GAATTCCGATCGACAAGGAACTGTATCCTTTAACTTCCC

TCA(G/A)ATC(A/C)CTCTTTGG-3' (55-mer pool) (SEQ ID

NO:24)

25) 5'-GTTAACGGATCCCAGCACTGACTAATTTATCTACTT(G/T)

(T/C)TCATTTCCTCC(A/T)AT-3'

(52-mer pool) (SEQ ID NO:25)

26) 5'-ATCTCTTACCTGTCCTATCTAACAGGCCAGGATTAA-3'

(36-mer) (SEQ ID NO:26)

27) 5'-GAATTCTCGCGACCACCATGGCGCGTTCAACGCTC-3'

(35-mer) (SEQ ID NO:27)

28) 5'-GTTAACAGATCTTCATGGCTCGTACTCTAT-3'

(30-mer) (SEQ ID NO:28)

29) 5'-GAATTCGCGCGCAAGCGGCCGCAACCCGGGAAAAGCTT

AAGCATGCAACCCGGGAAGAATTCAATCGCGAAA-3'

(72-mer) (SEQ ID NO:29)

30) 5'-GTTAACGCGCGCTTCTCGAGTTGCGGCCGCTTGCTAGCTT

AGATCTTTGGGCCCTTTCGCGATTGAATTCTT-3'

(72-mer) (SEQ ID NO:30)

31) 5'-GAATTCAAGCTTGGCCATTGCATACGTTGT-3'

(30-mer) (SEQ ID NO:31)

32) 5'-GTTAACGCATGCATAAGAAGCCAA-3'

(24-mer) (SEQ ID NO:32)

33) 5'-GAATTCGCATGCTCCCCTGCTCCGACCCGG-3'
     (30-mer) (SEQ ID NO: 33)

34) 5'-GTTAACGAATTCTCCTGCGGGGAGAAGCAG-3'
     (30-mer) (SEQ ID NO:34)

5   35) 5'-GAATTCAGATCTGCCATACCACATTTGTAG-3'
     (30-mer) (SEQ ID NO:35)

36) 5'-GTTAACGCTAGCTCCAGACATGATAAGATA-3'
     (30-mer) (SEQ ID NO:36)

37) 5'-GAATTCGCTAGCATCCCGCCCCTAACTCCG-3'
10   (30-mer) (SEQ ID NO:37)

38) 5'-GTTAACGTCGACGCAAAAGCCTAGGCCTCC-3'
     (30-mer) (SEQ ID NO:38)

39) 5'-GAATTCTCGCGAACAGTTGGCCCT-3'
     (24-mer) (SEQ ID NO:39)

15   40) 5'-GTTAACAGATCTTTACGCGAACGCGAAGTC-3'
     (30-mer) (SEQ ID NO:40)

41) 5'-GTTAACGAATTCTTGCAAAAGCTTTGCAAGATGGATA
     AAGTTTTTAGAAACTCCAGTAGGACTCC-3'
     (66-mer) (SEQ ID NO:41)

20   42) 5'-GAATTCTCGCGATCTAGACGTTCTACCTTTCTCTTCTT
     TTTTGGAGGAGTCCTACTGGAGTTT-3'
     (63-mer) (SEQ ID NO:42)

43) 5'-GTTAACGAATTCCCACCATGATTGAACAAGATGGA-5'
     (35-mer) (SEQ ID NO:43)

25   44) 5'-GAATTCAGATCTTCAGAAGAACTCGTCAAG-3'
     (30-mer) (SEQ ID NO:44)

45) 5'-CCCCGTGCCAAGAGTGACTACGTAAGTACCGCCTATAGA-3'
     (39-mer) (SEQ ID NO:45)

46) 5'-CTCTGCTTCTCCCCGCAGCTGGAGAATTCAATCGCGAAA-3'
30   (39-mer) (SEQ ID NO:46)

47) 5'-GTTAACGAATTCCCACCATGAACACGATTAACATC-5'
     (35-mer) (SEQ ID NO:47)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANTS: Capon, Daniel J. and Christos J. Petropoulous

    (ii) TITLE OF INVENTION: Compositions And Methods For Determining Anti-viral Drug Susceptibility And Resistance

    (iii)   NUMBER OF SEQUENCES: 47

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Cooper & Dunham LLP
        (B) STREET: 1185 Avenue of the Americas
        (C) CITY: New York
        (D) STATE: New York
        (E) COUNTRY: United States
        (F) ZIP: 10036

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (viii)ATTORNEY/AGENT INFORMATION:
        (A) NAME: White, John P.
        (B) REGISTRATION NUMBER: 28,678
        (C) REFERENCE/DOCKET NUMBER: 50130-A-PCT/JPW/AKC

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: 212-278-0400
        (B) TELEFAX: 212-391-0526

(2) INFORMATION FOR SEQ ID NO:1:

    (I) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

-158-

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGTGAATTAG CCCTTCCACC CGGGTCGAGC TTGGCGTAAT CA
              42

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 42 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTGTTGGGAA GGGCGATCTC TAGATGCTAG AGATTTTCCA CA
              42

(2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 44 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTCCTCCTCC AAGTCTGAGC GGCCGCCTTT AGCATCTGAT GCAC
              44

(2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 44 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTCCTCCTCC AAGTCTGAGC GGCCGCCATA TGGTGTTTTA CTAA
              44

(2) INFORMATION FOR SEQ ID NO:5:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 36 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTCTAACCA GAGAGACCCG GTTCACTAAA CGAGCT
            36

    (2) INFORMATION FOR SEQ ID NO:6:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 32 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAATTCGCGG CCGCAATTCC GCCCCTCTCC CT
            32

    (2) INFORMATION FOR SEQ ID NO:7:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 32 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTTAACGCGG CCGCGATATA GTTCCTCCTT TC
            32

    (2) INFORMATION FOR SEQ ID NO:8:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 32 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

-160-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAATTCTCGC GACCATGGAA GACGCCAAAA AC
                32

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 36 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTTAACAGAT CTCTCGAGTT ACAATTTGGA CTTTCC
                36

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 56 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGACGGGCAC ACACTACTTA ATACGACTCA CTATAGGGTG AAGCACTCAA
GGCAAG   56

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AAGAGTGACC TGAGGGAAGT TAACGGATAC AGTTCCTTGT CT
                42

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TCCAGCACTG ACTAATTTGT CGACTTGTTC ATTTCCTCCA AT
         42

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TAACGCCTAT TCTGCTATGC CGACACCCAA TTCTGAAAAT GG
         42

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAGGATACAG TTCCTTGTCG ATCGGCTCCT GCTTCTGAGG GG
         42

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

      (xi). SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTAAAAATAG TACTTTCCGG ATCCCAGCAC TGACTAATTT AT
               42

5

(2) INFORMATION FOR SEQ ID NO:16:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 42 base pairs

10
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

15
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTAGCTCCTT CGGTCCTCCA ATCGTTGTCA GAAGTAAGTT GG
              42

20

(2) INFORMATION FOR SEQ ID NO:17:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 42 base pairs

25
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

30
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTCCCAGATA AGTGCCAAGG ATTCGTTCAC TAATCGAATG GA
              42

35

(2) INFORMATION FOR SEQ ID NO:18:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 33 base pairs

40
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

45
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAATTCGTTA ACTTCCCTCA GATCACTCTT TGG
              33

50

(2) INFORMATION FOR SEQ ID NO:19:

-163-

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTTAACGTCG ACTTGTTCAT TTCCTCCAAT
          30

(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 55 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GAATTCCGAT CGACAAGGAA CTGTATCCTT TAACTTCCCT CAGATCACTC TTTGG
          55

(2) INFORMATION FOR SEQ ID NO:21:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 52 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTTAACGGAT CCCAGCACTG ACTAATTTAT CTACTTGTTC ATTTCCTCCA AT
          52

(2) INFORMATION FOR SEQ ID NO:22:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

BEST AVAILABLE COPY

-164-

```
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GAATTCGTTA ACTTCCCTCA RATCMCTCTT TGG
            33

(2) INFORMATION FOR SEQ ID NO:23:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTTAACGTCG ACTTKYTCAT TTCCTCCWAT
            30

(2) INFORMATION FOR SEQ ID NO:24:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 55 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GAATTCCGAT CGACAAGGAA CTGTATCCTT TAACTTCCCT CARATCMCTC TTTGG
            55

(2) INFORMATION FOR SEQ ID NO:25:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 52 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTTAACGGAT CCCAGCACTG ACTAATTTAT CTACTTKYTC ATTTCCTCCW AT
            52

(2) INFORMATION FOR SEQ ID NO:26:
```

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 36 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: DNA (genomic)

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATCTCTTACC TGTCCTATCT AACAGGCCAG GATTAA
          36

   (2) INFORMATION FOR SEQ ID NO:27:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 35 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: DNA (genomic)

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GAATTCTCGC GACCACCATG GCGCGTTCAA CGCTC
          35

   (2) INFORMATION FOR SEQ ID NO:28:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 30 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: DNA (genomic)

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GTTAACAGAT CTTCATGGCT CGTACTCTAT
          30

   (2) INFORMATION FOR SEQ ID NO:29:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 72 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GAATTCGCGC   GCAAGCGGCC   GCAACCCGGG   AAAAGCTTAA   GCATGCAACC
CGGGAAGAAT   60

TCAATCGCGA AA
                72

(2) INFORMATION FOR SEQ ID NO:30:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 72 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTTAACGCGC   GCTTCTCGAG   TTGCGGCCGC   TTGCTAGCTT   AGATCTTTGG
GCCCTTTCGC   60

GATTGAATTC TT
                72

(2) INFORMATION FOR SEQ ID NO:31:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 30 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GAATTCAAGC TTGGCCATTG CATACGTTGT
                30

(2) INFORMATION FOR SEQ ID NO:32:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 24 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

-167-

GTTAACGCAT GCATAAGAAG CCAA
                24

(2) INFORMATION FOR SEQ ID NO:33:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 30 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GAATTCGCAT GCTCCCCTGC TCCGACCCGG
                30

(2) INFORMATION FOR SEQ ID NO:34:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 30 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GTTAACGAAT TCTCCTGCGG GGAGAAGCAG
                30

(2) INFORMATION FOR SEQ ID NO:35:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 30 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GAATTCAGAT CTGCCATACC ACATTTGTAG
                30

(2) INFORMATION FOR SEQ ID NO:36:
    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 30 base pairs
       (B) TYPE: nucleic acid

-168-

         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GTTAACGCTA GCTCCAGACA TGATAAGATA
          30

(2) INFORMATION FOR SEQ ID NO:37:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 30 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GAATTCGCTA GCATCCCGCC CCTAACTCCG
          30

(2) INFORMATION FOR SEQ ID NO:38:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 30 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GTTAACGTCG ACGCAAAAGC CTAGGCCTCC
          30

(2) INFORMATION FOR SEQ ID NO:39:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 24 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GAATTCTCGC GAACAGTTGG CCCT
                24

(2) INFORMATION FOR SEQ ID NO:40:

      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GTTAACAGAT CTTTACGCGA ACGCGAAGTC
                30

(2) INFORMATION FOR SEQ ID NO:41:

      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 66 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GTTAACGAAT TCTTGCAAAA AGCTTTGCAA GATGGATAAA GTTTTTAGAA
ACTCCAGTAG   60

GACTCC
                66

(2) INFORMATION FOR SEQ ID NO:42:

      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 63 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GAATTCTCGC GATCTAGACG TTCTACCTTT CTCTTCTTTT TTGGAGGAGT
CCTACTGGAG TTT   63

-170-

(2) INFORMATION FOR SEQ ID NO:43:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 35 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GTTAACGAAT TCCCACCATG ATTGAACAAG ATGGA
        35

(2) INFORMATION FOR SEQ ID NO:44:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GAATTCAGAT CTTCAGAAGA ACTCGTCAAG
        30

(2) INFORMATION FOR SEQ ID NO:45:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 39 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CCCCGTGCCA AGAGTGACTA CGTAAGTACC GCCTATAGA
        39

(2) INFORMATION FOR SEQ ID NO:46:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 39 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

5   CTCTGCTTCT CCCCGCAGCT GGAGAATTCA ATCGCGAAA
                39

    (2) INFORMATION FOR SEQ ID NO:47:

10   (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 35 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear
15
            (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

20   GTTAACGAAT TCCCACCATG AACACGATTA ACATC
                35

What is claimed is:

1.   A method for determining susceptibility for an anti-viral drug comprising:
   (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell;
   (b) culturing the host cell from (a);
   (c) measuring expression of the indicator gene in a target host cell; and
   (d) comparing the expression of the indicator gene from (c) with the expression of the indicator gene measured when steps (a)-(c) are carried out in the absence of the anti-viral drug,
   wherein a test concentration of the anti-viral drug is present at steps (a)-(c); at steps (b)-(c); or at step (c).

2.   The method of claim 1 wherein the resistance test vector comprises DNA of a genomic viral vector.

3.   The method of claim 1 wherein the resistance test vector comprises DNA of a subgenomic viral vector.

4.   The method of claim 1 wherein the resistance test vector comprises DNA of a retrovirus.

5.   The method of claim 1 wherein the resistance test vector comprises DNA of HIV.

6.   The method of claim 1 wherein the resistance test vector comprises DNA encoding *vif, vpr, tat, rev, vpu,* and *nef*.

7.   The method of claim 1 wherein the patient-derived segment comprises a functional viral sequence.

8.   The method of claim 1 wherein the patient-derived segment encodes one protein that is the target of an anti-viral drug.

9.   The method of claim 1 wherein the patient-derived segment encodes two or more proteins that are the target of an anti-viral drug.

10. The method of claim 1 wherein the patient-derived segment comprises a retroviral gene.

11. The method of claim 1 wherein the patient-derived segment comprises an HIV gene.

12. The method of claim 1 wherein the patient-derived segment comprises an HIV *gag-pol* gene.

13. The method of claim 1 wherein the indicator gene is a functional indicator gene and the host cell is a resistance test vector host cell including the additional step of infecting the target host cell with resistance test vector viral particles using filtered supernatants from said resistance test vector host cells.

14. The method of claim 1 wherein the indicator gene is a non-functional indicator gene.

15. The method of claim 14 wherein the host cell is a packaging host cell/resistance test vector host cell.

35   16. The method of claim 15 wherein the culture is by

co-cultivation.

17.   The method of claim 15 wherein the target host cell is infected with resistance test vector viral particles using filtered supernatants from said packaging host cell/resistance test vector host cells.

18.   The method of claim 1 wherein the indicator gene is a luciferase gene.

19.   The method of claim 1 wherein the indicator gene is an *E. coli lacZ* gene.

20.   The method of claim 15 wherein the packaging host cell/resistance test vector host cell is a human cell.

21.   The method of claim 15 wherein the packaging host cell/resistance test vector host cell is a human embryonic kidney cell.

22.   The method of claim 15 wherein the packaging host cell/resistance test vector host cell is a 293 cell.

23.   The method of claim 1 wherein the target host cell is a human T cell.

24.   The method of claim 1 wherein the target host cell is a human T cell leukemia cell line.

25.   The method of claim 1 wherein the target host cell is a Jurkat cell line.

26.   The method of claim 1 wherein the target host cell is a H9 cell line.

27. The method of claim 1 wherein the target host cell is
     a CEM cell line.

28. A resistance test vector comprising a patient-derived
     segment and an indicator gene.

29. The resistance test vector of claim 28 wherein the
     patient-derived segment is one gene.

30. The resistance test vector of claim 28 wherein the
     patient-derived segment is two or more genes.

31. The resistance test vector of claim 28 wherein the
     patient-derived segment comprises a retroviral gene.

32. The resistance test vector of claim 28 wherein the
     patient-derived segment comprises an HIV gene.

33. The resistance test vector of claim 28 wherein the
     patient-derived segment comprises an HIV *gag-pol* gene.

34. The resistance test vector of claim 28 wherein the
     indicator gene is a functional indicator gene.

35. The resistance test vector of claim 28 wherein the
     indicator gene is a non-functional indicator gene.

36. The resistance test vector of claim 28 wherein the
     indicator gene is a luciferase gene.

37. A packaging host cell transfected with a resistance
     test vector.

38. The packaging host cell of claim 37 that is a mammalian
     host cell.

39. The packaging host cell of claim 37 that is a human host cell.

40. The packaging host cell of claim 37 that is a human embryonic kidney cell.

41. The packaging host cell of claim 37 that is 293 cells.

42. The packaging host cell of claim 37 that is a human hepatoma cell line.

43. The packaging host cell of claim 37 that is HepG2.

44. The packaging host cell of claim 37 that is Huh7.

45. A method for determining susceptibility for an anti-viral drug comprising:
    (a) introducing a resistance test vector comprising a patient-derived segment and a nonfunctional indicator gene into a host cell;
    (b) culturing the host cell from (a);
    (c) measuring expression of the indicator gene in a target host cell; and
    (d) comparing the expression of the indicator gene from (c) with the expression of the indicator gene measured when steps (a)-(c) are carried out in the absence of the anti-viral drug,
    wherein a test concentration of the anti-viral drug is present at steps (a)-(c); at steps (b)-(c); or at step (c).

46. The method of claim 45 wherein the resistance test vector comprises DNA of a genomic viral vector.

-177-

47. The method of claim 45 wherein the resistance test
    vector comprises DNA of a subgenomic viral vector.

48. The method of claim 45 wherein the resistance test
    vector comprises DNA of a retrovirus.

49. The method of claim 45 wherein the resistance test
    vector comprises DNA of HIV.

50. The method of claim 45 wherein the resistance test
    vector comprises DNA encoding *vif*, *vpr*, *tat*, *rev*, *vpu*,
    and *nef*.

51. The method of claim 45 wherein the patient-derived
    segment encodes one protein.

52. The method of claim 45 wherein the patient-derived
    segment encodes two or more proteins.

53. The method of claim 45 wherein the patient-derived
    segment comprises a retroviral gene.

54. The method of claim 45 wherein the patient-derived
    segment comprises an HIV gene.

55. The method of claim 45 wherein the patient-derived
    segment comprises an HIV *gag-pol* gene.

56. The method of claim 45 wherein the indicator gene is a
    luciferase gene.

57. The method of claim 45 wherein the host cell is a
    packaging host cell.

58. The method of claim 45 wherein the packaging host cell

is a human cell.

59. The method of claim 45 wherein the packaging host cell
    is a human embryonic kidney cell.

60. The method of claim 45 wherein the packaging host cell
    is a 293 cell.

61. The packaging host cell of claim 45 that is a human
    hepatoma cell line.

62. The packaging host cell of claim 45 that is HepG2.

63. The packaging host cell of claim 45 that is Huh7.

64. The method of claim 45 wherein the nonfunctional
    indicator gene comprises a permuted promoter.

65. The method of claim 45 wherein the nonfunctional
    indicator gene comprises a permuted coding region.

66. The method of claim 45 wherein the nonfunctional
    indicator gene comprises an inverted intron.

67. The method of claim 45 wherein the host cell and target
    cell are the same cell.

68. The method of claim 45 wherein the target cell is a
    human cell.

69. The method of claim 45 wherein the target cell is a
    human T cell.

70. The method of claim 57 wherein the target host cell is
    infected with resistance test vector viral particles

-179-

using filtered supernatants from said packaging host
cell/resistance test vector host cell.

71.   The method of claim 57 wherein said culture is by
      co-cultivation.

72.   A method for determining anti-viral drug resistance in
      a patient comprising:
      (a)   developing   a   standard   curve   of   drug
            susceptibility for an anti-viral drug;
      (b)   determining anti-viral drug susceptibility in the
            patient according to the method of claim 1; and
      (c)   comparing the anti-viral drug susceptibility in
            step (b) with the standard curve determined in
            step (a), wherein a decrease in anti-viral
            susceptibility   indicates   development   of
            anti-viral drug resistance in the patient.

73.   A method for determining anti-viral drug resistance in
      a patient comprising:
      (a)   developing   a   standard   curve   of   drug
            susceptibility for an anti-viral drug;
      (b)   determining anti-viral drug susceptibility in the
            patient according to the method of claim 45; and
      (c)   comparing the anti-viral drug susceptibility in
            step (b) with the standard curve determined in
            step (a), wherein a decrease in anti-viral
            susceptibility   indicates   development   of
            anti-viral drug resistance in the patient.

74.   A method for determining anti-viral drug resistance in
      a patient comprising:
      (a)   determining anti-viral drug susceptibility in the
            patient at a first time according to the method
            of claim 1, wherein the patient-derived segment

is obtained from the patient at about said time;

    (b)   determining anti-viral drug susceptibility of the
same patient at a later time; and

    (c)   comparing the anti-viral drug susceptibilities
determined in step (a) and (b), wherein a
decrease in anti-viral drug susceptibility at the
later time compared to the first time indicates
development or progression of anti-viral drug
resistance in the patient.

75. A method for determining anti-viral drug resistance in
a patient comprising:

    (a)   determining anti-viral drug susceptibility in the
patient at a first time according to the method
of claim 45, wherein the patient-derived segment
is obtained from the patient at about said time;

    (b)   determining anti-viral drug susceptibility of the
same patient at a later time; and

    (c)   comparing the anti-viral drug susceptibilities
determined in steps (a) and (b), wherein a
decrease in anti-viral drug susceptibility at the
later time compared to the first time indicates
development or progression of anti-viral drug
resistance in the patient.

76. The method of claim 1 wherein the resistance test
vector comprises DNA of a hepadnavirus.

77. The method of claim 1 wherein the resistance test
vector comprises DNA of HBV.

78. The method of claim 1 wherein the resistance test
vector comprises DNA encoding C, P, and X.

79. The method of claim 1 wherein the patient-derived

segment comprises a P gene.

80. The method of claim 1 wherein the patient-derived segment comprises an HBV gene.

81. The method of claim 1 wherein the patient-derived segment comprises an HBV RT gene.

82. The method of claim 1 wherein the patient-derived segment comprises an HBV DNA polymerase gene.

83. The resistance test vector of claim 28 comprising an indicator gene viral vector and a packaging vector said indicator gene viral vector comprising an indicator gene and said packaging vector comprising a patient-derived segment.

84. The resistance test vector of claim 28 wherein the patient-derived segment comprises a hepadnaviral gene.

85. The resistance test vector of claim 28 wherein the patient-derived segment comprises an HBV gene.

86. The resistance test vector of claim 28 wherein the patient-derived segment comprises an HBV P gene.

87. The resistance test vector of claim 28 wherein the patient-derived segment comprises an RT/DNA polymerase gene.

88. A method for evaluating the biological effectiveness of a candidate anti-viral drug compound comprising:
    (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell;

-182-

(b)   culturing the host cell from step (a);

(c)   measuring expression of the indicator gene in a
      target host cell; and

(d)   comparing the expression of the indicator gene
      from step (c) with the expression of the indicator
      gene measured when steps (a)-(c) are carried out
      in the absence of the candidate anti-viral drug
      compound,

wherein a test concentration of the candidate
anti-viral drug compound is present at steps (a)-(c);
at steps (b)-(c); or at step (c).

89.   The method of claim 88 wherein the resistance test
      vector comprises DNA of a retrovirus.

90.   The method of claim 88 wherein the resistance test
      vector comprises DNA of HIV.

91.   The method of claim 88 wherein the resistance test
      vector comprises DNA of a hepadnavirus.

92.   The method of claim 88 wherein the resistance test
      vector comprises DNA of HBV.

93.   The method of claim 88 wherein the resistance test
      vector comprises DNA encoding HIV *gag-pol*.

94.   The method of claim 88 wherein the resistance test
      vector comprises DNA encoding HBV P protein.

95.   The method of claim 88 wherein the patient-derived
      segment encodes one protein.

96.   The method of claim 88 wherein the patient-derived
      segment encodes two or more proteins.

97.   The method of claim 88 wherein the patient-derived
      segment comprises a retroviral gene.

98.   The method of claim 88 wherein the patient-derived
      segment comprises an HIV gene.

99.   The method of claim 88 wherein the patient-derived
      segment comprises a hepadnaviral gene.

100. The method of claim 88 wherein the patient-derived
      segment comprises an HBV gene.

101. A   method   for   determining   susceptibility   for   an
      anti-viral drug comprising:
      (a) introducing a resistance test vector comprising a
           patient-derived segment and an indicator into a
           host cell;
      (b) culturing the host cell from (a);
      (c) measuring the indicator in a target host cell;
           and
      (d) comparing the measurement of the indicator from
           (c) with the measurement of the indicator when
           steps (a)-(c) are carried out in the absence of
           the anti-viral drug,
      wherein a test concentration of the anti-viral drug is
      present at steps (a)-(c); at steps (b)-(c); or at step
      (c).

102. The method of claim 101 wherein the indicator comprises
      a DNA structure.

103. The method of claim 101 wherein the indicator comprises
      a RNA structure.

-184-

104. A method for evaluating the biological effectiveness of
     a candidate anti-viral drug compound comprising:
     (a) introducing a resistance test vector comprising a
          patient-derived segment and an indicator into a
5   host cell;
     (b) culturing the host cell from step (a);
     (c) measuring the indicator in a target host cell; and
     (d) comparing the measurement of the indicator from
          step (c) with the measurement of the indicator
10   measured when steps (a)-(c) are carried out in the
          absence of the candidate anti-viral drug compound,
     wherein a test concentration of the candidate
     anti-viral drug compound is present at steps (a)-(c);
     at steps (b)-(c); or at step (c).
15

105. The method of claim 104 wherein the indicator comprises
     a DNA structure.

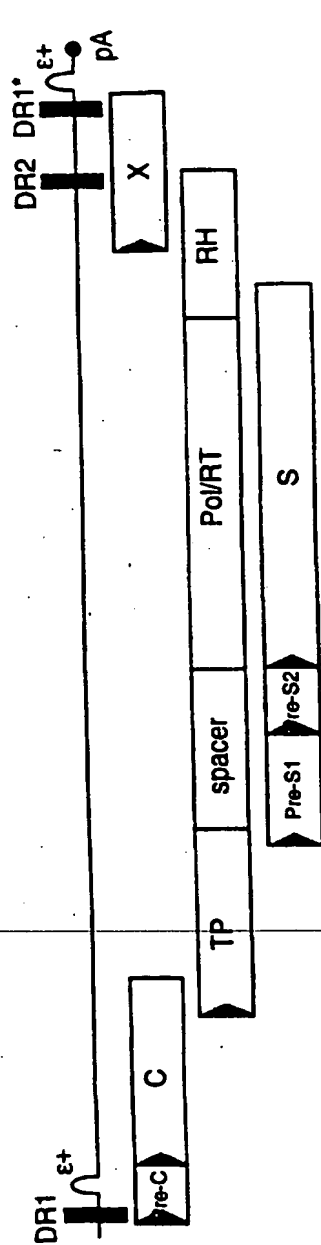106. The method of claim 105 wherein the indicator comprises
20   a RNA structure.

FIG. 1A
FIG. 1B
FIG. 1C
FIG. 1D
FIG.1E

FIG. 2A

FIG. 2B

FIG. 2C
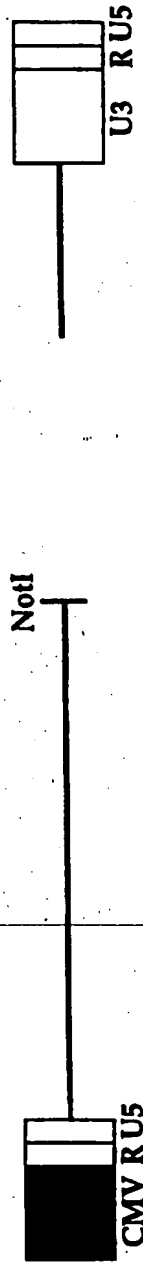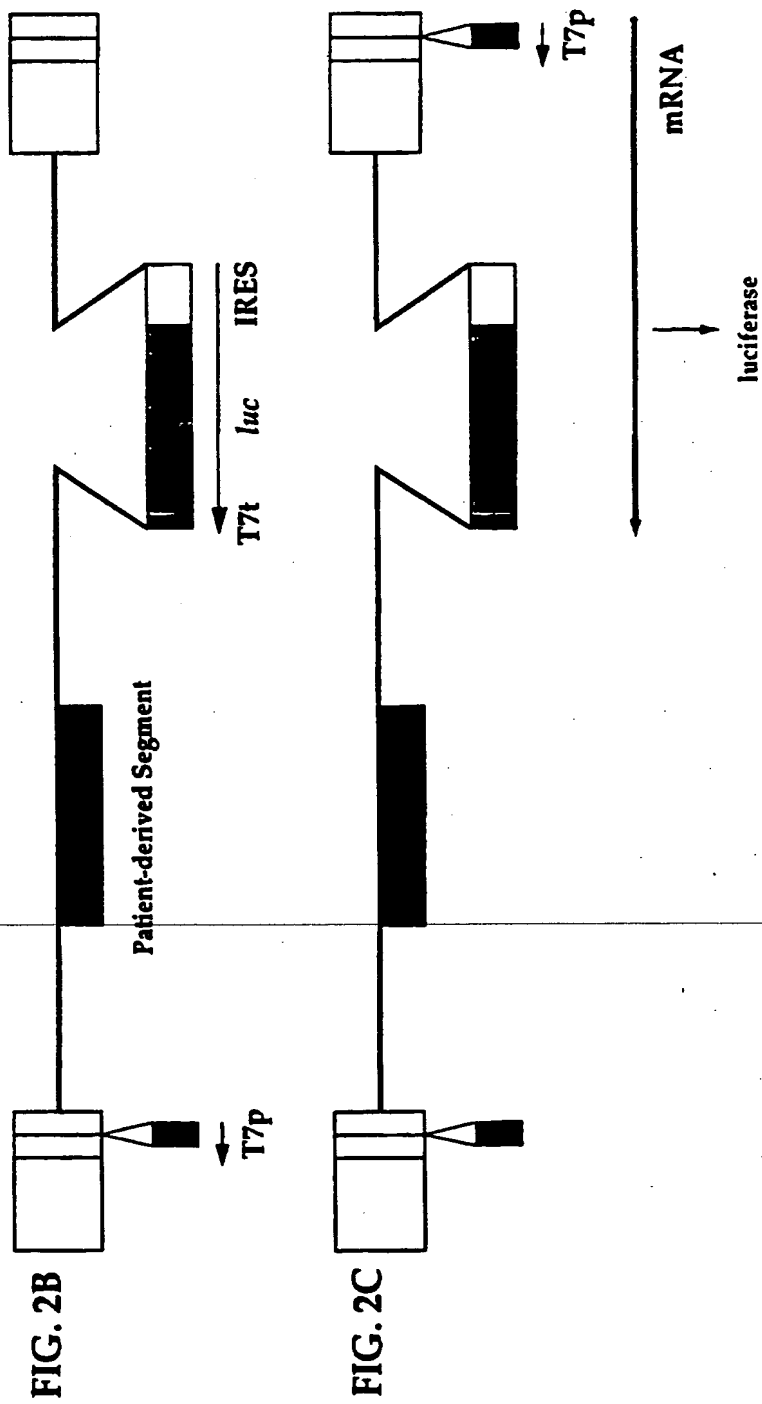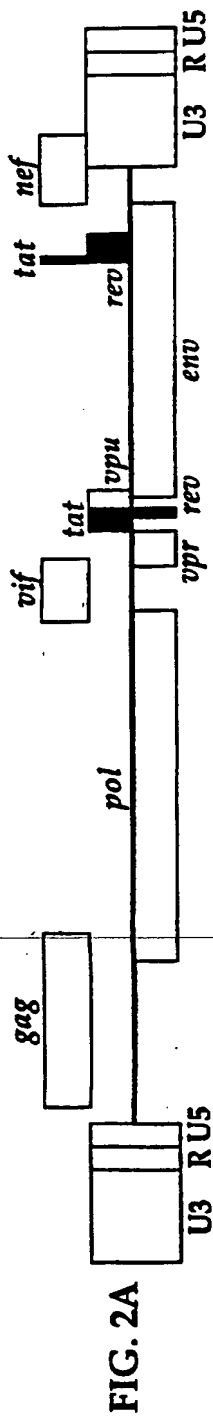
3/22
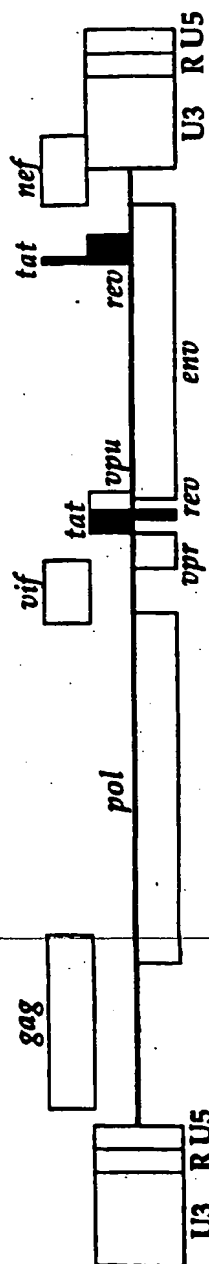
FIG.3A

FIG.3B

FIG.3C
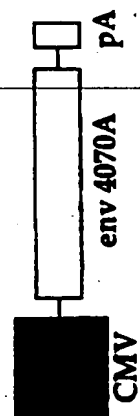
FIG.3D
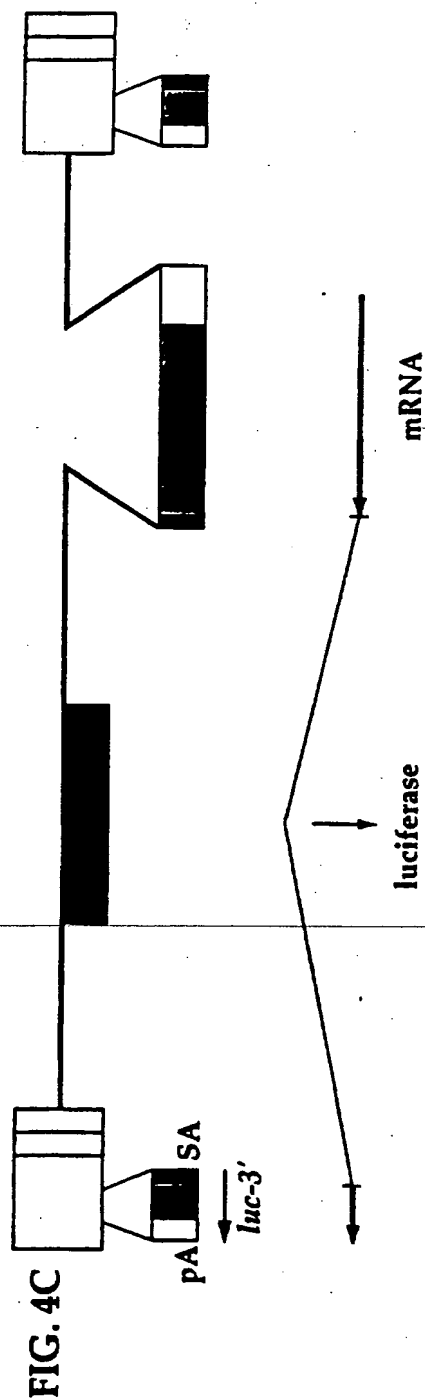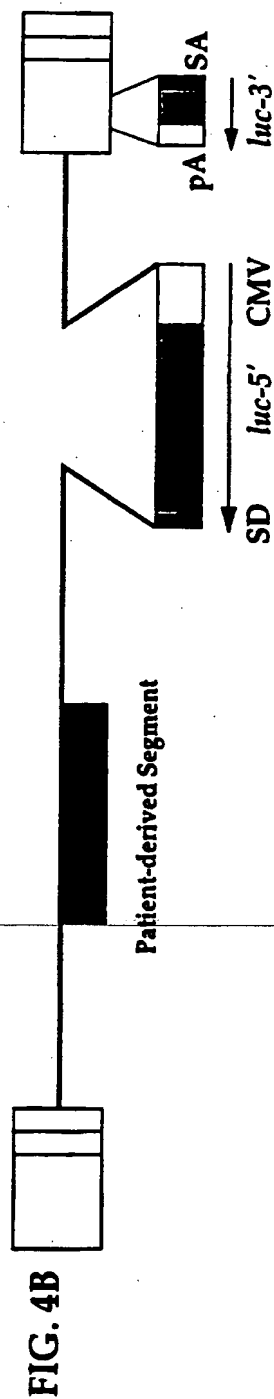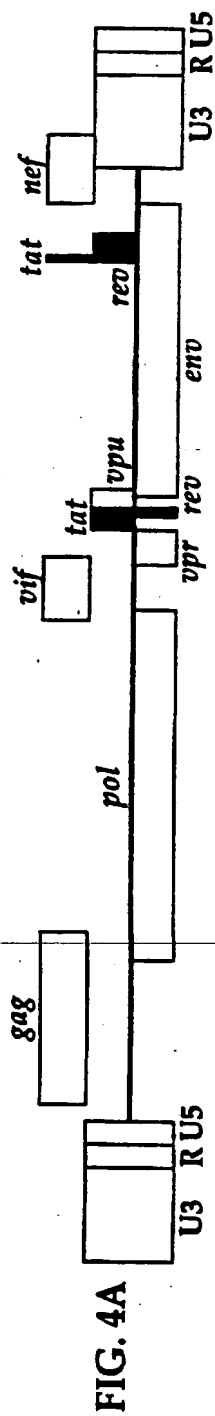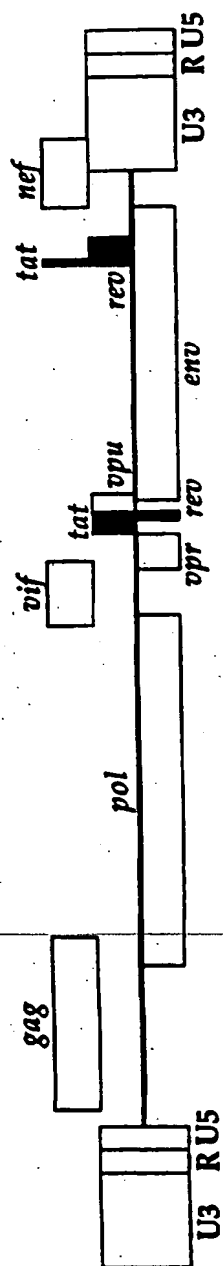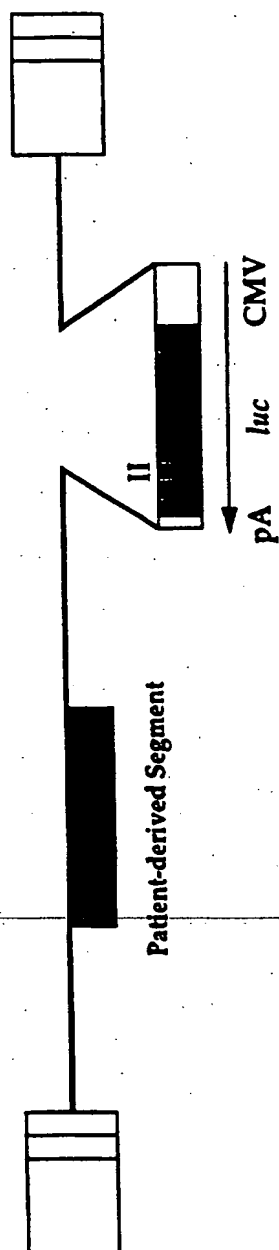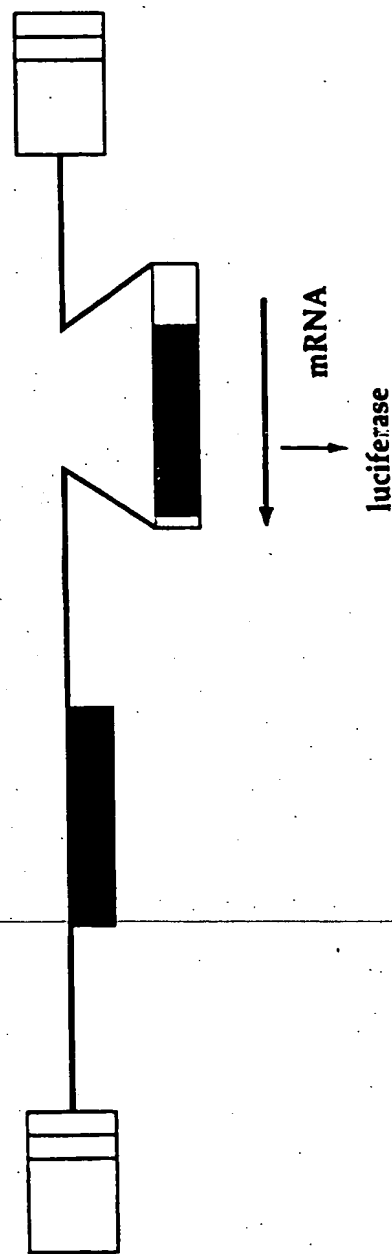
FIG. 4A

FIG. 4B

FIG. 4C

5/22

FIG. 5A

FIG. 5B

FIG. 5C
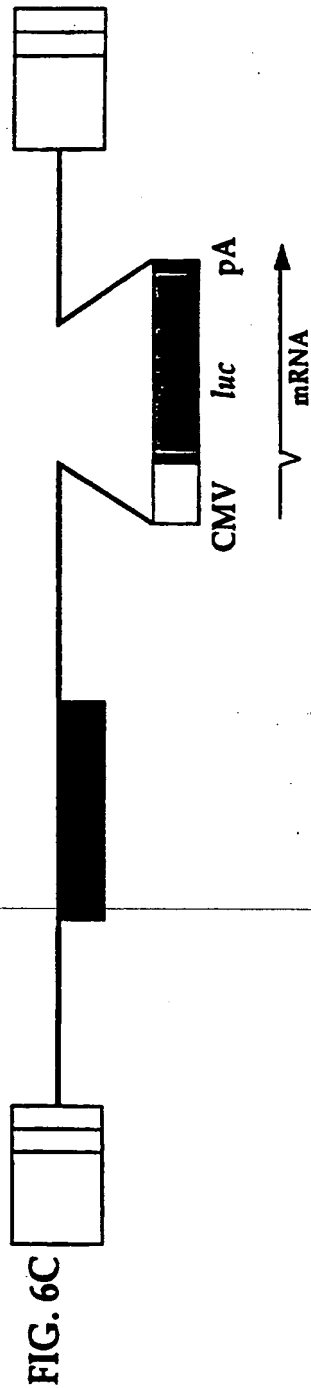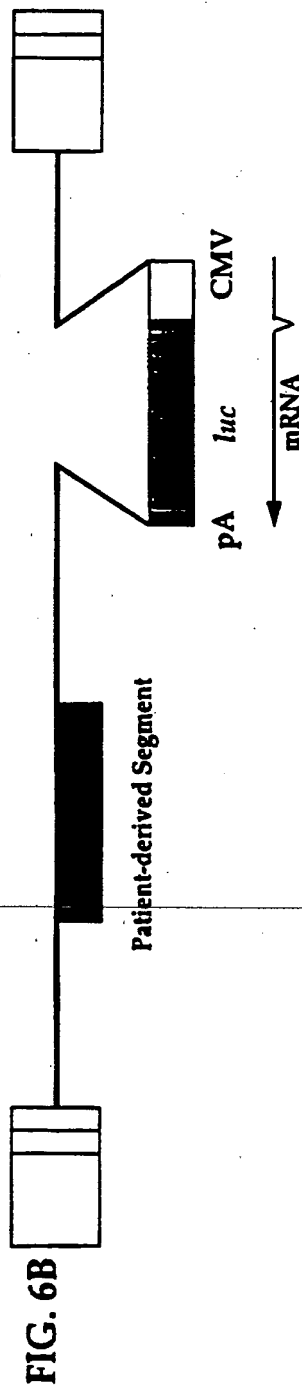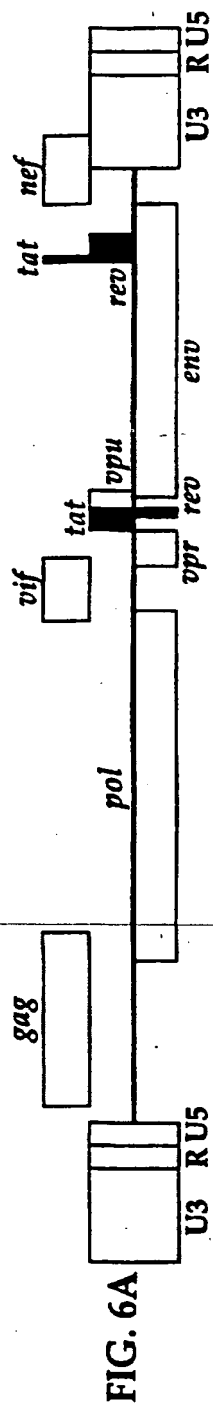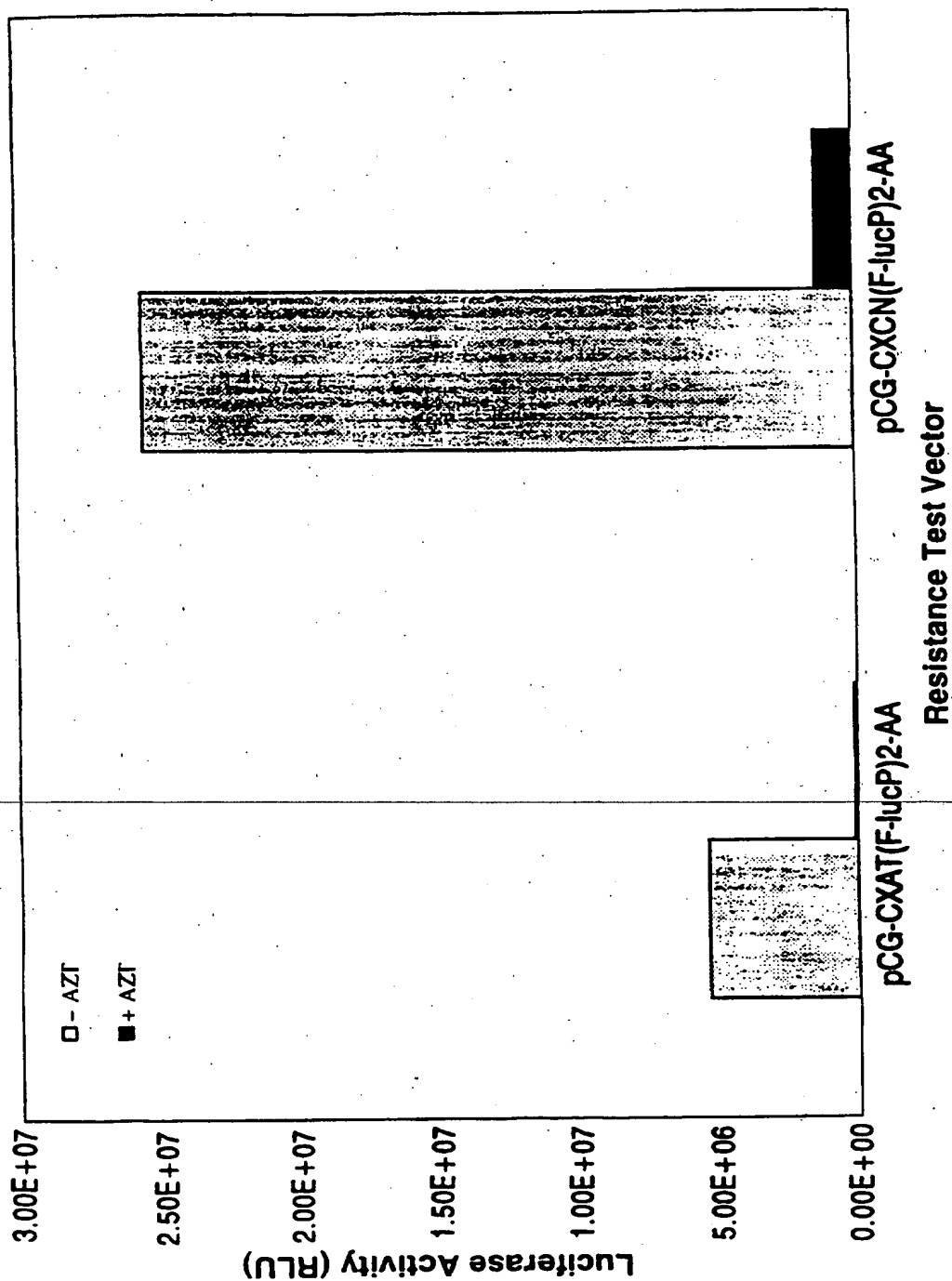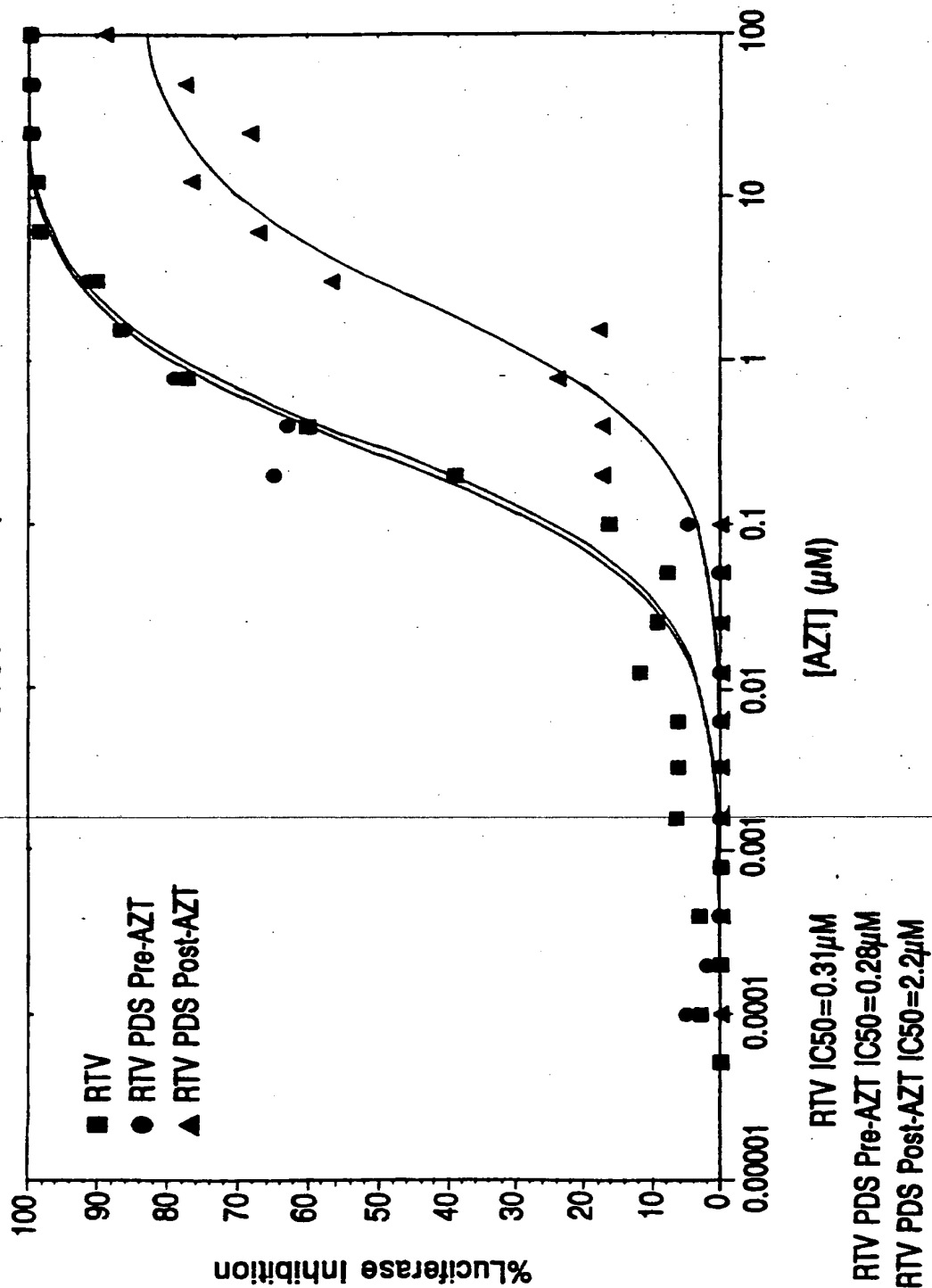
FIG. 6A

FIG. 6B

FIG. 6C

FIG. 7A

FIG. 7B

RTV IC50=0.31μM
RTV PDS Pre-AZT IC50=0.28μM
RTV PDS Post-AZT IC50=2.2μM

FIG. 7C

[Nevirapine] (µM)

%Luciferase Inhibition

IC50=0.04µM

FIG. 7D

FIG. 8A

FIG. 8B

FIG. 8C

splice junction

FIG. 8D

FIG. 8E

Fig. 8F

Fig. 8G

Fig. 8H

Fig. 9A

Fig. 9B

Fig. 9C

Fig. 9D

Fig. 9E

Fig. 9F

Fig. 9G

FIG. 10A

FIG. 10B

FIG. 10C

Fig. 10D

Fig. 10E

Fig. 11A

Fig. 11B

Fig. 11C

Fig. 11D

Fig. 11E
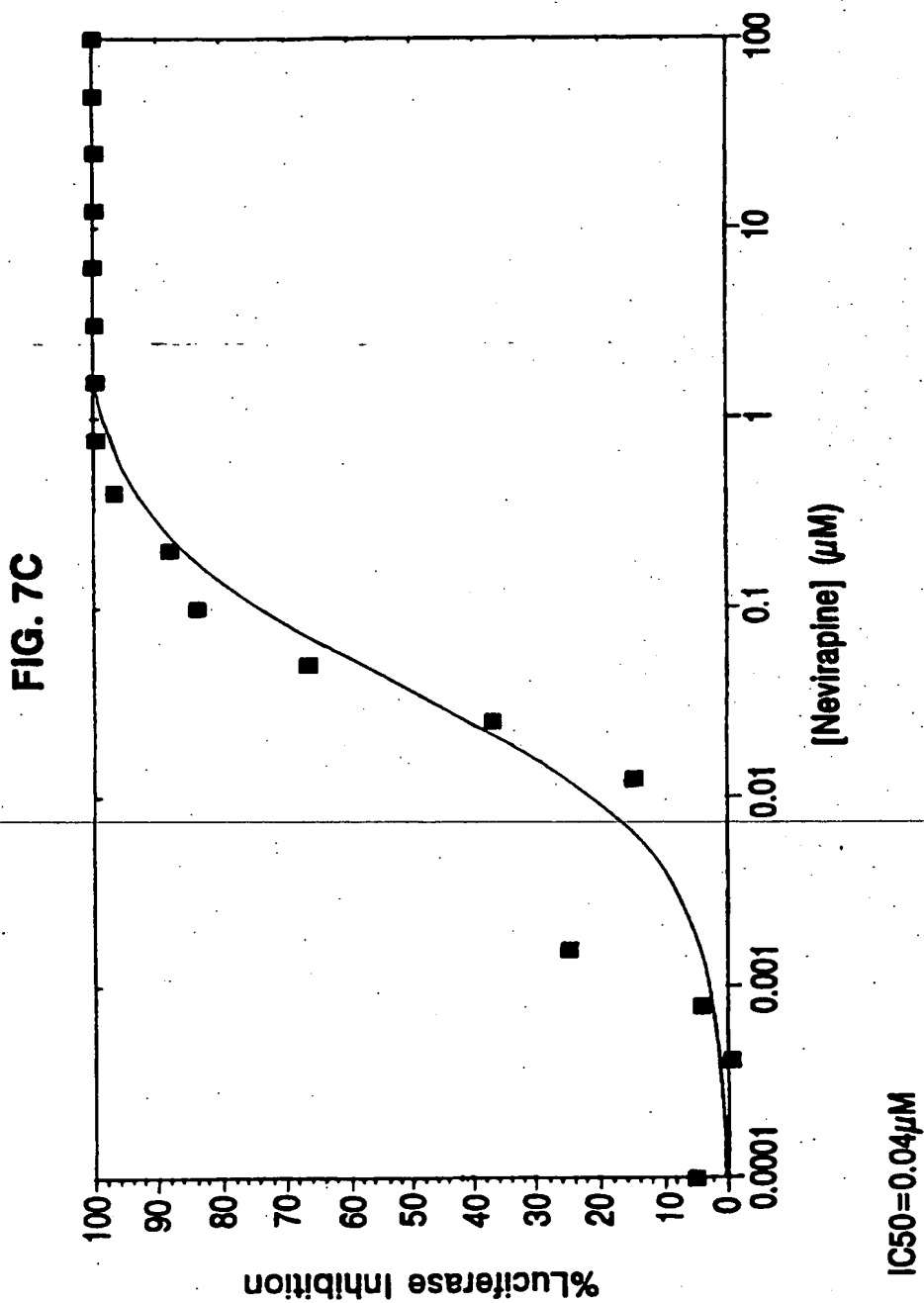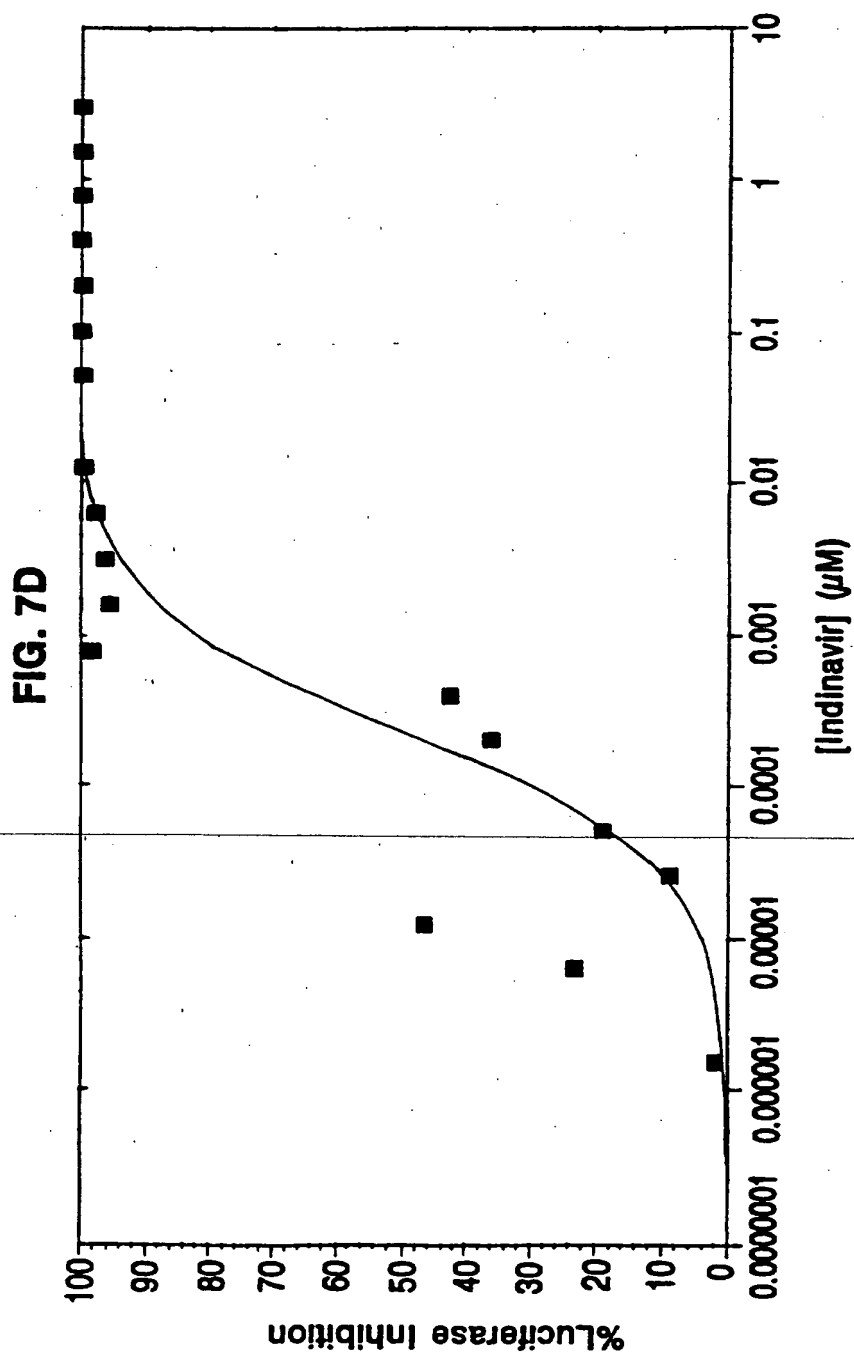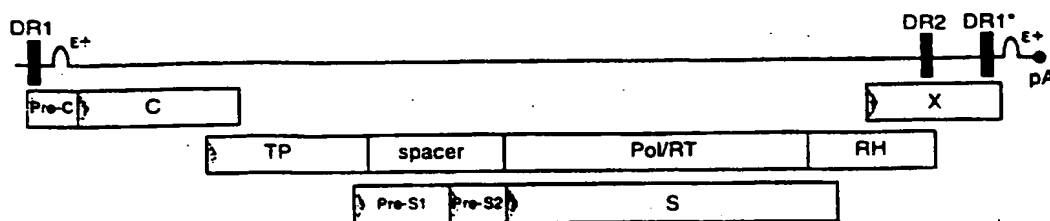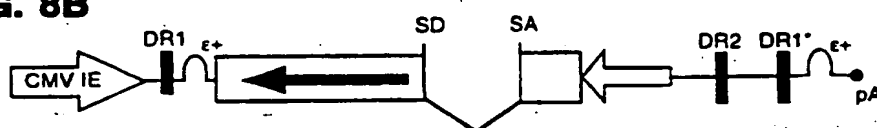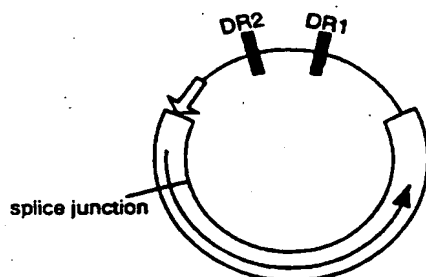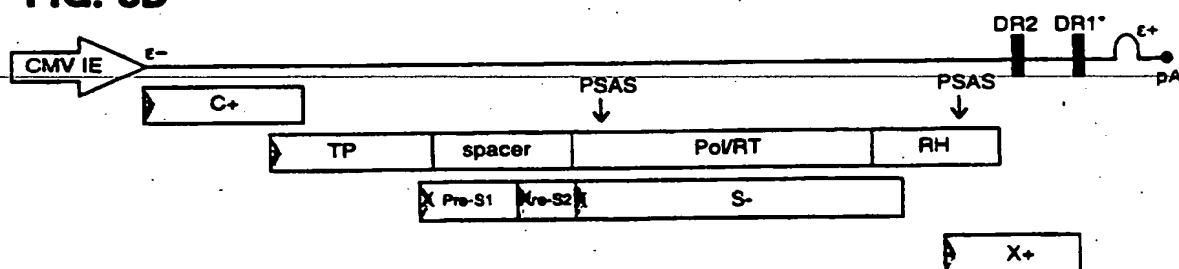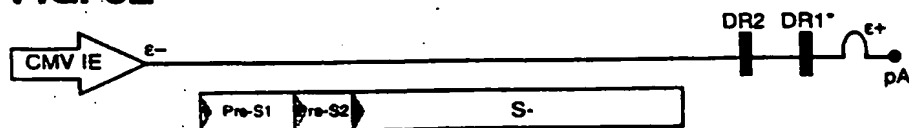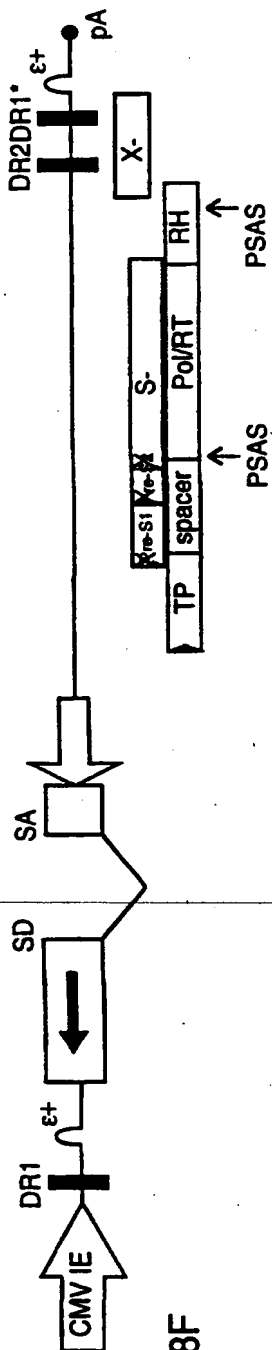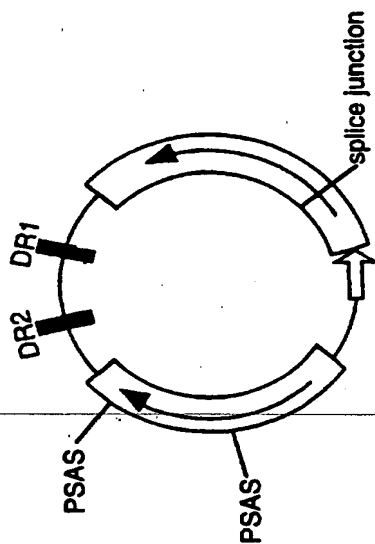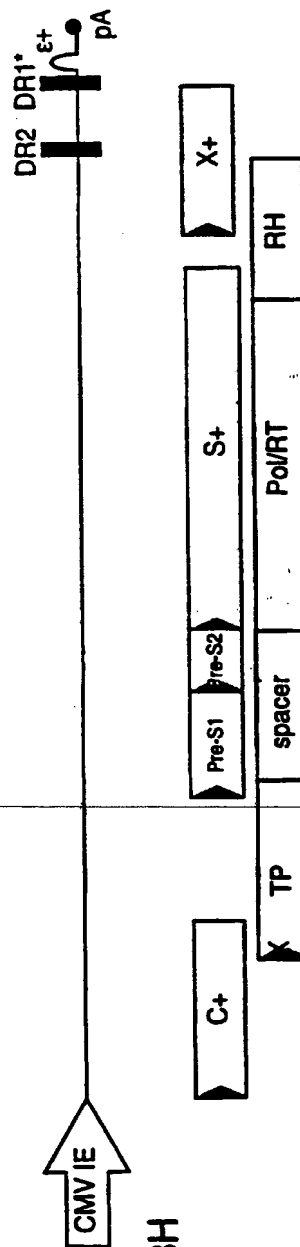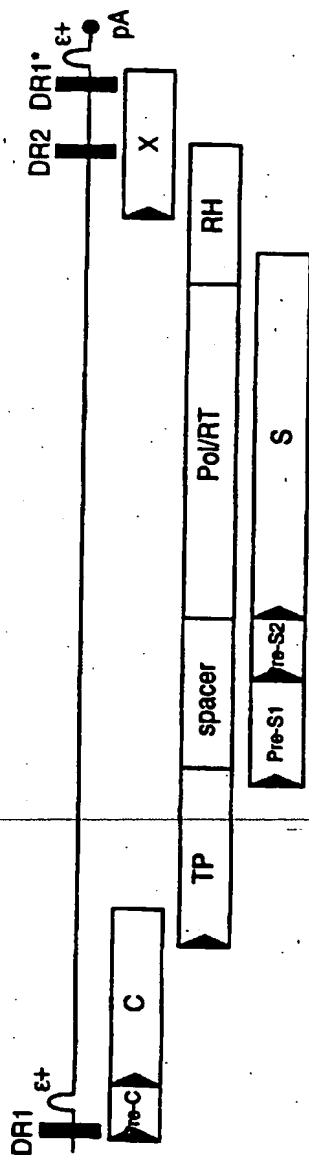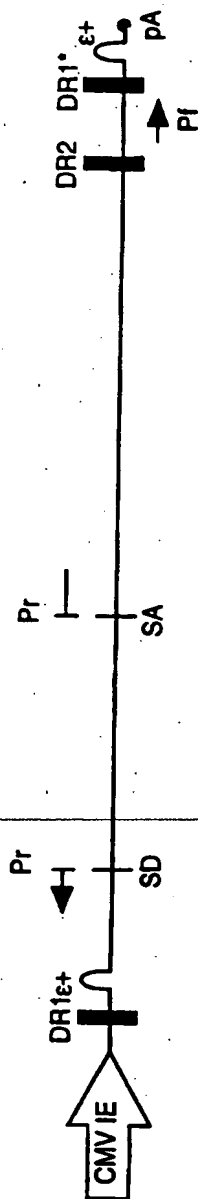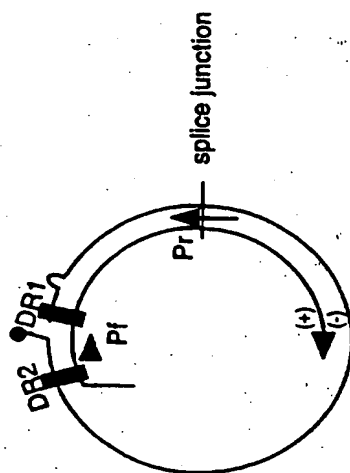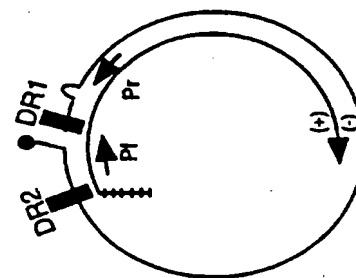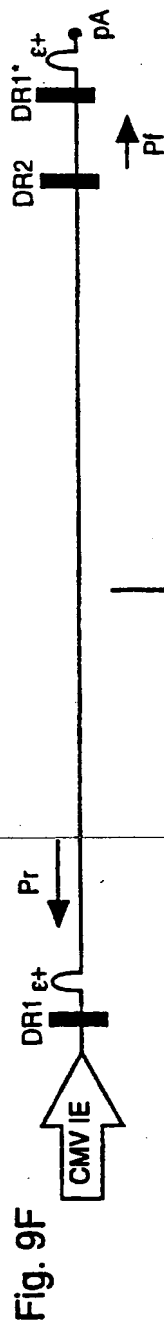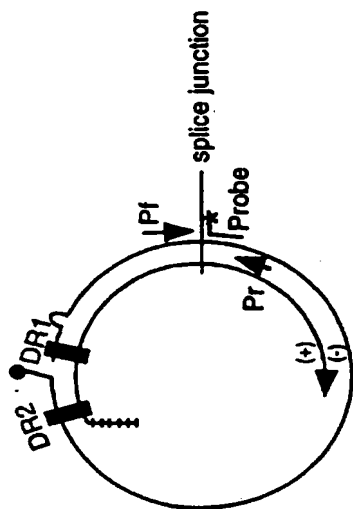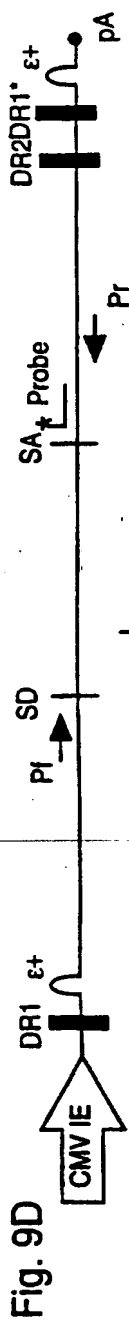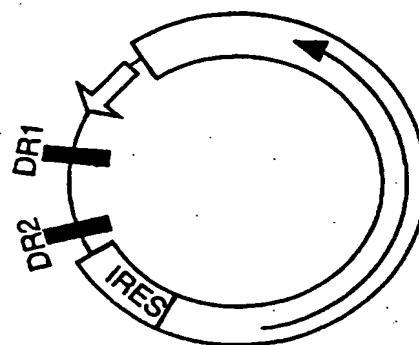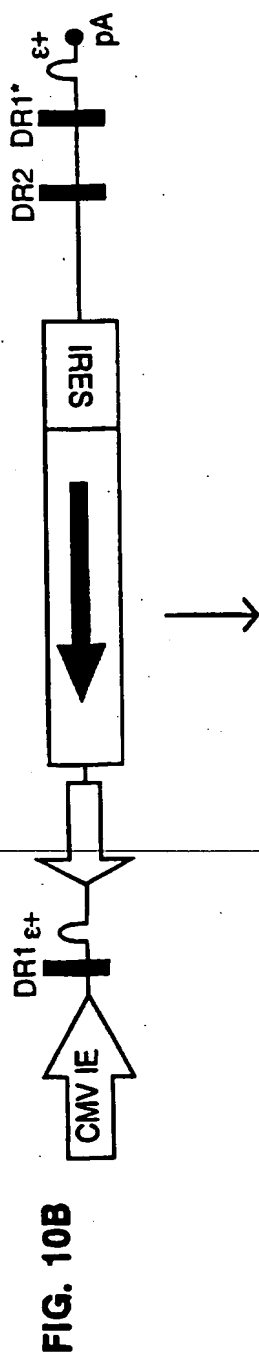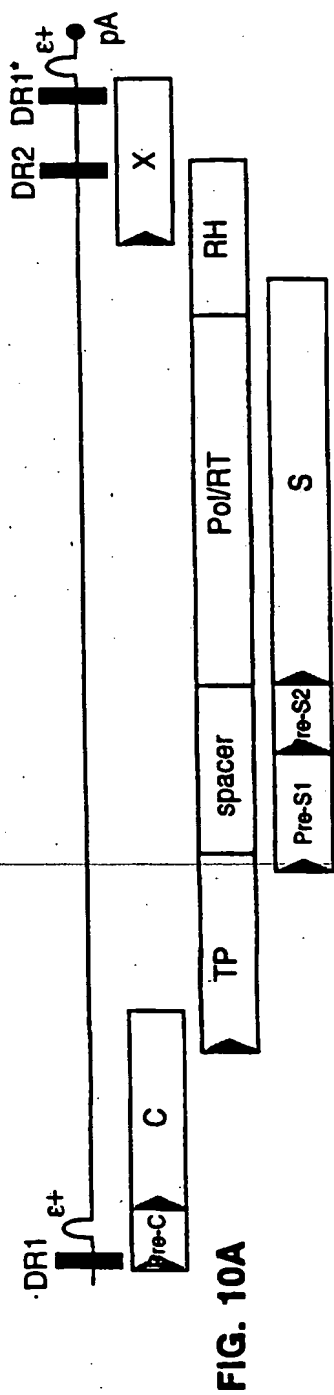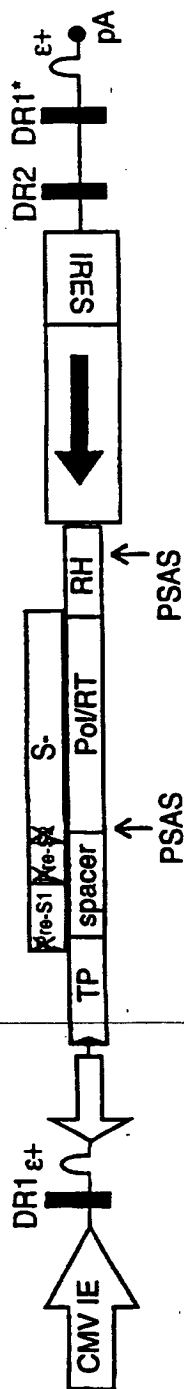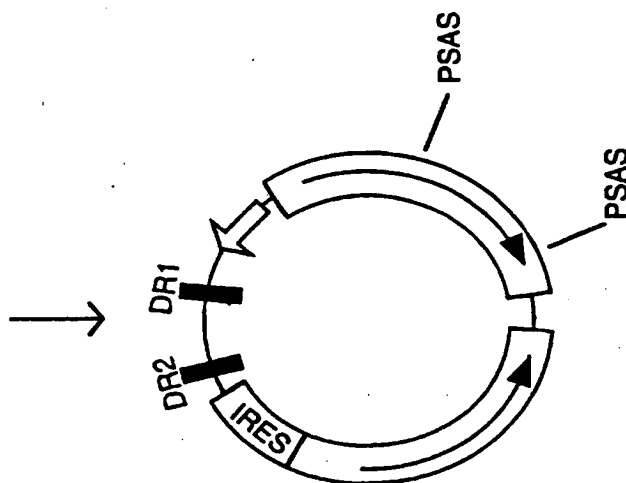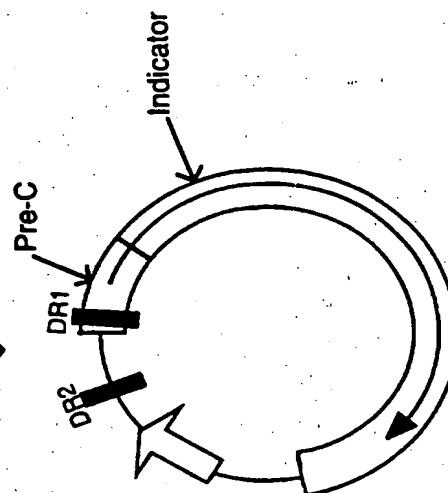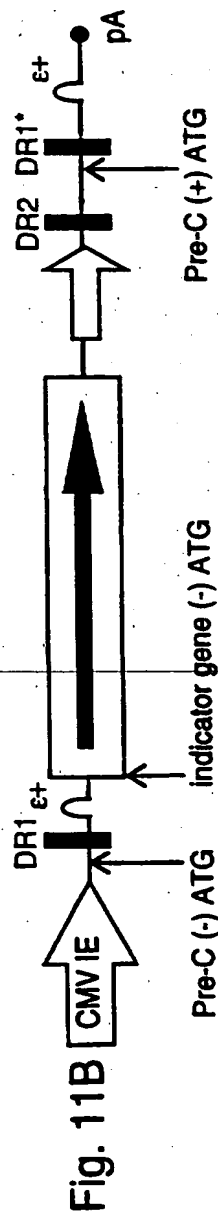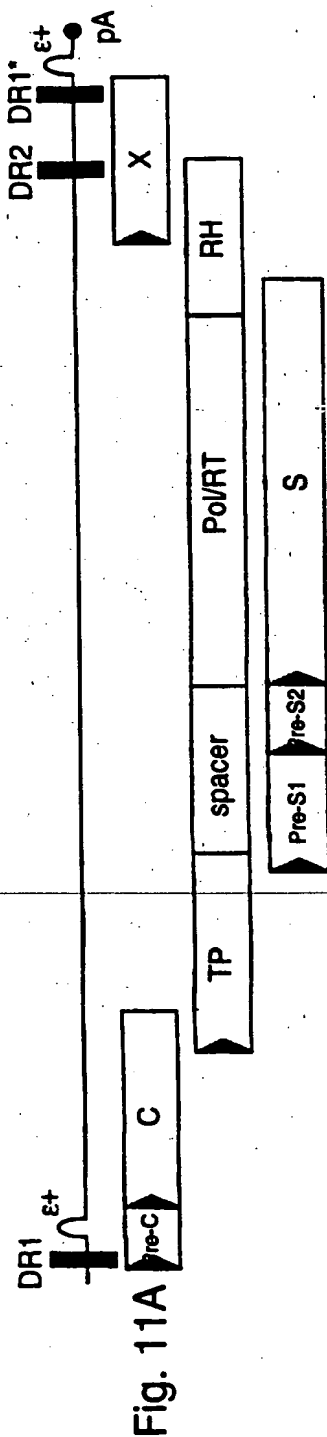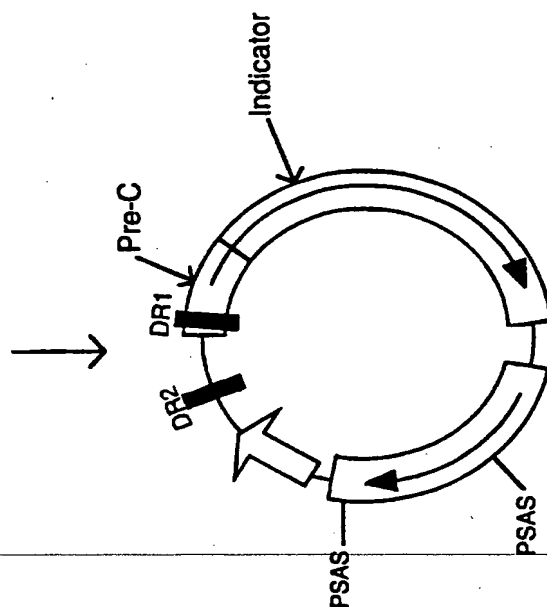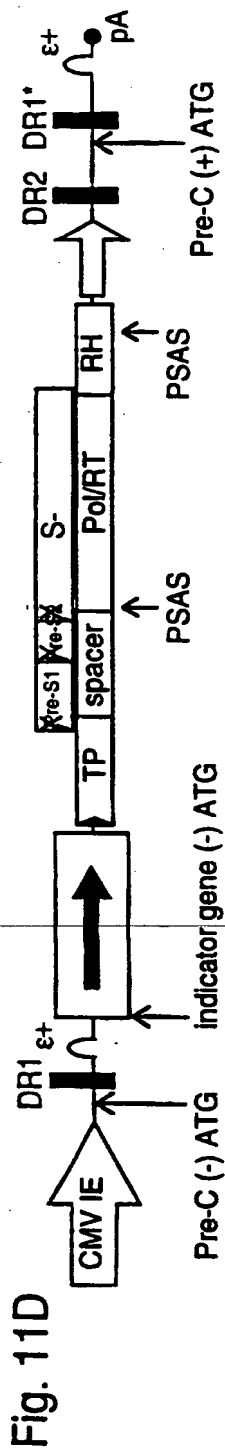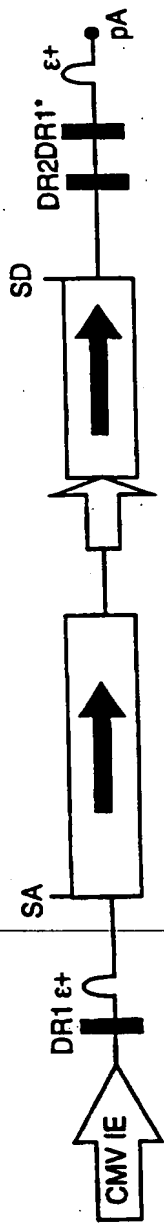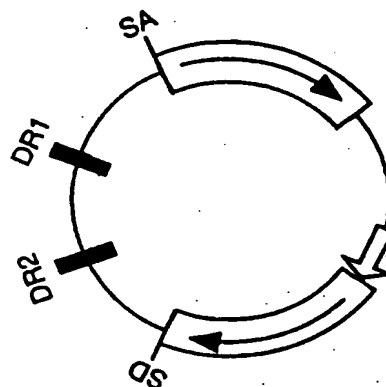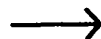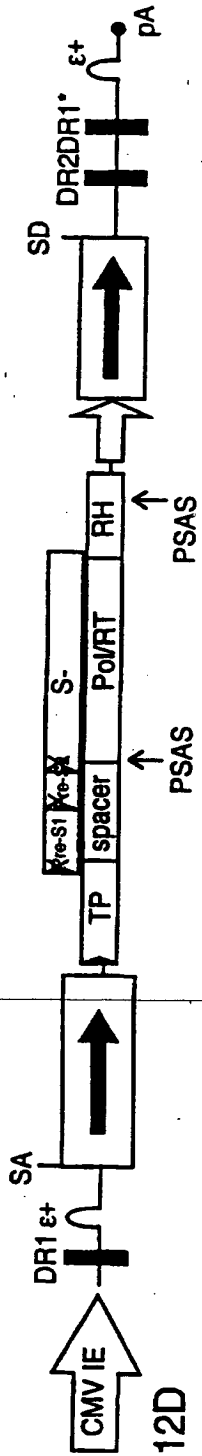
Fig. 12A
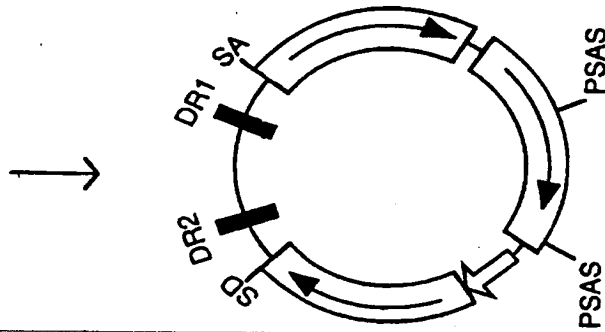
Fig. 12B

Fig. 12C

Fig. 12D

FIG. 12E
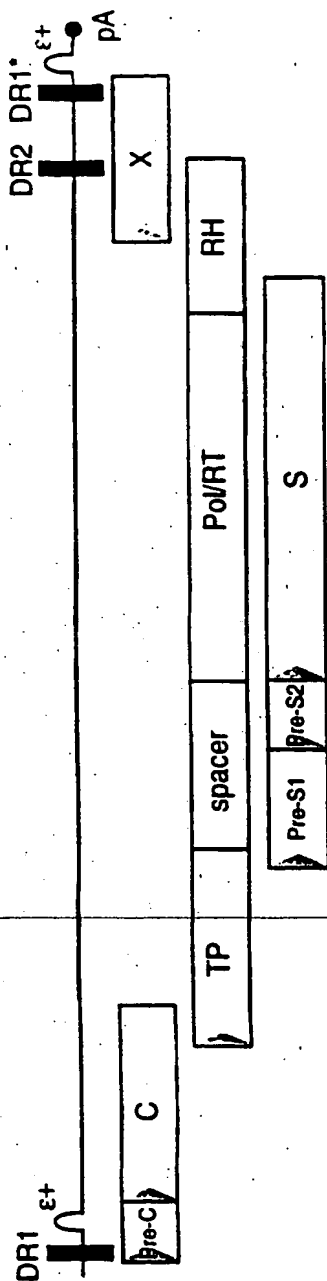
Fig. 13A

Fig. 13B

Fig. 13C

Fig. 13D
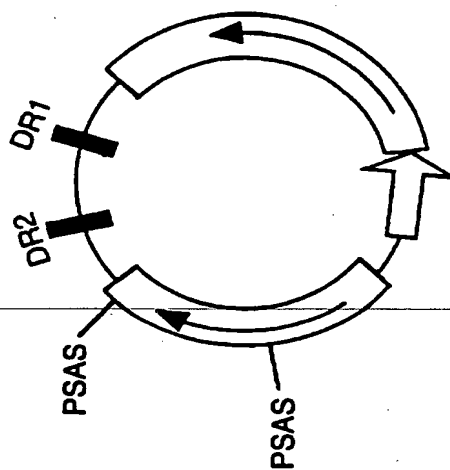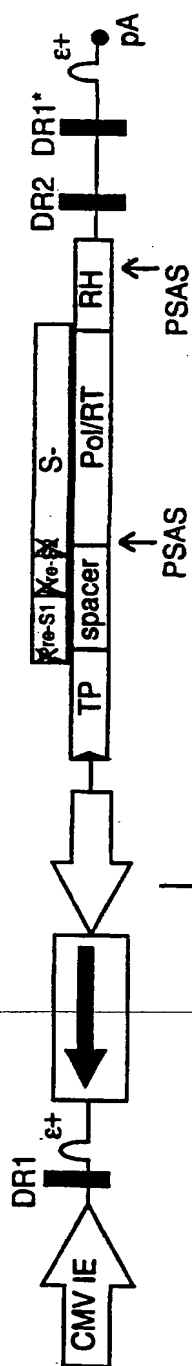
Fig. 13E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01609

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/02; C12N 15/49, 15/64
US CL :435/6, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN (Medline, Biosis, CAPLUS, EMBASE)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SALTARELLI. M.J. et al. The CAEV *tat* Gene *Trans*-activates the Viral LTR and Is Necessary for Efficient Viral Replication. Virology. 1993, Vol. 197, pages 35-44, see entire document.	28-44
A	KELLAM. P. et al. Recombinant Virus Assay: a Rapid, Phenotypic Assay for Assessment of Drug Susceptibility of Human Immunodeficiency Virus Type 1 Isolates. Antimicrobial Agents and Chemotherapy. January 1994, Vol. 38, pages 23-30.	1-106

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 APRIL 1997	**2 1 APR 1997**

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MATTHEW LATIMER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

THIS PAGE BLANK (USPTO)